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PRINCIPAL INVESTIGATOR: Garth F. Hall Ph.D.

CONTRACTING ORGANIZATION: University of Massachusetts Lowell, Lowell, MA 01854

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Garth F. Hall Ph.D.

Email: Garth_Hall@uml.edu

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14. ABSTRACT

The goal of this project is to identify CSF biomarkers for Alzheimer's Disease (AD) based on known cellular events in AD pathogenesis related to 1) tau secretion and 2) the synergy between AD and repeated traumatic brain injury (chronic traumatic encephalopathy or CTE). The project is intended to increase our understanding of the underlying causes of both CTE and AD as well as improve our ability to diagnose the presence and severity of these conditions in a timely manner. Our approach is based on observations that tau isoforms lacking exon 2-3 (E2- tau) are secreted more readily than E2+ isoforms in cell culture (Kim 2010) and that secreted microvesicles (exosomes) containing tau and other proteins are identifiable in the cerebrospinal fluid (CSF) of patients in the early stages of AD. The Aims of the project are to 1) characterize the cellular distribution of E2+ and E2- tau isoforms in fixed brain tissue from early AD and CTE patients, to quantify their presence in CSF and brain homogenate exosomal fractions and to identify proteins associated with tau (and particularly E2- and E2+ tau) in these fractions in the context of AD/CTE cytopathogenesis. The second Aim of this project is to directly test the effects of overexpressing E2- and E2+ tau isoforms on tau distribution, secretion and the recruitment of other proteins to exosomes in neuronal cell lines (by mass spectrometry). The presence and distribution of proteins identified as potential key players in AD/CTE-associated tau secretion will then be tested in human brain and CSF.

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1 Introduction

The goal of this project is to identify CSF biomarkers for Alzheimer's Disease (AD) based on known cellular events in AD pathogenesis related to 1) tau secretion and 2) the synergy between AD and repeated traumatic brain injury (chronic traumatic encephalopathy or CTE). The project is intended to increase our understanding of the underlying causes of both CTE and AD as well as improve our ability to diagnose the presence and severity of these conditions in a timely manner. Our approach is based on observations that tau isoforms lacking exon 2-3 (E2- tau) are secreted more readily than E2+ isoforms in cell culture (Kim 2010) and that secreted microvesicles (exosomes) containing tau and other proteins are identifiable in the cerebrospinal fluid (CSF) of patients in the early stages of AD. The Aims of the project are to 1) characterize the cellular distribution of E2+ and E2- tau isoforms in fixed brain tissue from early AD and CTE patients, to quantify their presence in CSF and brain homogenate exosomal fractions and to identify proteins associated with tau (and particularly E2- and E2+ tau) in these fractions in the context of AD/CTE cytopathogenesis. The second Aim of this project is to directly test the effects of overexpressing E2- and E2+ tau isoforms on tau distribution, secretion and the recruitment of other proteins to exosomes in neuronal cell lines (by mass spectrometry). The presence and distribution of proteins identified as potential key players in AD/CTE-associated tau secretion will then be tested in human brain and CSF.

2 Keywords

Tau isoforms

Alzheimer's Disease pathogenesis

AD links to CTE

Alzheimer's Disease CSF biomarkers

Bioinformatic analysis

Exosome

3 Overall Project Summary

Aim 1: Sensitivity and specificity of the N-Terminal Tau fragment as a biomarker

Overview:
Key results from the completed work after 1 year are as follows:

- Findings from the bioinformatics-based analyses of Aims 1a and 2a have suggested specific mechanisms by which normally non-exosomal proteins may be recruited to exosomes in early AD (see attached MS).
- Immunohistochemical staining of E2- and E2+ tau in AD brain show that E2- tau appears more peripherally distributed in the somata and dendrites than E2+ tau and may be concentrated in areas with high GFAP immunolabel (Figure 1), such as might be expected with senile plaques (**1**). This pattern suggests that E2- tau may accumulate as the result of Abeta – tau interactions associated with inflammation.

A) Exosome analysis of CSF & brain homogenates

We have made major progress on understanding the bioinformatics of the CSF exosomal proteome in early AD and have achieved a detailed characterization of the recruitment pathways of normally non-exosomal proteins to exosomes. This has been done in comparison with ischemia-associated dementia (MID/VaD) controls. This work is described in detail in the MS ready for submission (attached) and its likely importance is summarized below in the Accomplishments/Impacts section. Briefly, we have analyzed the total of 3818 and 1992 discrete proteins identified in Braak stage 3-4 (i.e. early) AD and demented control samples (respectively) in terms of a) whether the proteins were identified as upregulated/downregulated in limbic cortical regions in early/moderate AD in online datasets (Antonell 2013, Blalock 2004, 2011), b) whether the proteins were listed as members of the extracellular vesicular exosome (EVE) GO term and c) the extent to which the proteins have been identified (on String 9.1) as having physical interactions with tau and beta amyloid. Our results can be summarized as follows: The non-exosomal proteins recruited to early AD CSF exosomes can be divided into 2 "recruitment pathways"; one of which (the specific pathway) involves upregulated cellular functions, specific interactions of individual proteins with tau/Abeta and affects signaling pathways inflammation and cell cycle control. The other recruitment pathway involves downregulated functions in AD; neuron-specific structures/functions and protein turnover mechanisms in particular and does not appear to depend upon physical interactions with tau/Abeta. This work has been complicated somewhat by the periodic updating of EVE GO term, which has expanded from several dozen members (as of last year) to 1535 members this March and 2396 members as of September 1 this year. This is no doubt due to the very high degree of interest in and research into exosomal functions during 2014. Other Go terms have been checked periodically and appear to be much more stable (fortunately!). We have also expanded somewhat the range of AD-associated cellular functions as relevant literature has been identified by us or has been published; this is particularly relevant to the addition of inflammation associated GO terms to our analyses as it became clear that a significant enrichment of such terms is present in the AD-associated CSF exosomal proteome. More time and effort was devoted to this aspect of the project than was originally expected because the results offer a detailed context for the interpretation of E2- vs E2+ tau data – the bioinformatic datasets in particular. This is evident in

the preliminary analysis of exosomal proteomes associated with E2- vs E2+ tau constructs inSH-SY5Y cells described below.

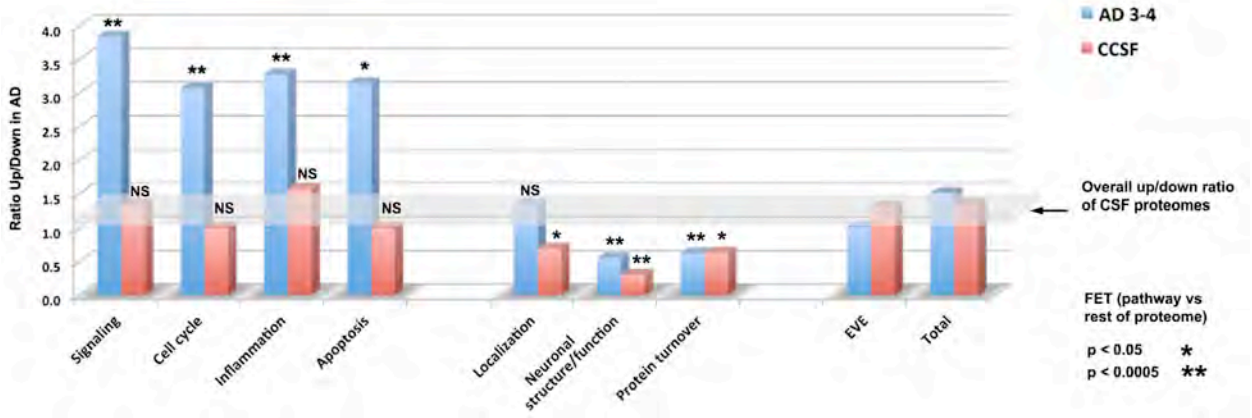

Figure 1 Upregulated (leftmost 4 bar pairs) and downregulated pathways (center 3 bar pairs) in AD are clearly distinguishable in the early AD (AD 3-4) exosomal proteome. Upregulated pathways are clearly uniquely affected in AD and not ischemia-associated dementia controls (CCSF), whereas downregulated pathways are less AD specific. This figure is part of Figure 2a in the attached MS and is described more fully there. FET = Fisher's Exact Test, NS = not significant.

Analysis of the exosome fraction from hippocampal homogenates has been less straightforward than that of CSF samples, possibly because of technical issues outlined in Problems section (below). As mentioned, we will be reanalyzing the AD and control homogenates using more sharply defined purification criteria than used previously (i.e. longer centrifugations times and sucrose gradient based purifications) and repeating the mass spectrometry step. This will be particularly important in light of preliminary results from the hippocampal homogenates suggesting that later stages of AD (Braak stage 5 and (especially) 6) are associated with a significant increase in downregulated proteins reaching the exosomal pathway (see the Q1 technical progress report). A preliminary GO term analysis of the hippocampal and CSF exosome fraction proteomes of individual AD patients with high E2-/E2+ tau ratios in their CSF samples is given in the Appendix (attached Excel file).

B) Immunohistochemistry on paraffin-embedded brain sections

Despite the problems identified in the overview, significant progress, much of it technical, has been made with E2- and E2+ immunohistochemistry studies of a small number of AD and CTE cases. In the AD cases, we have got consistent results with the E2- and E2+ mAbs (9A1 and DC39). These indicate that E2- tau is distributed in a less cytoskeletal pattern than is E2+ tau in pyramidal somata and dendrites, and is more frequently present on punctate bodies in the neuropil that appear to be synaptic terminals. Overall, the E2- pattern is more distributed to the cell periphery in a perimembranous pattern, and in many cases appears to be present in the interstitial space. A preliminary quantitative analysis (see the Q1 Technical Report for this project) showed that E2- immunolabel is more frequently present in neurites and small punctate bodies in the neuropil than is E2+ immunolabel; This tendency can be readily seen in the higher magnification panels of Figure 2. A particularly striking feature of the E2- immunostaining is its tendency to show patchy areas of increased immunostaining that resemble senile plaques (Figure 2C). This is particularly interesting in light of the bioinformatic analysis of the exosomal fraction proteins associated with E2- tau isoform expression in SH-SY5Y cells, which is associated with

downregulated in AD GO terms for synaptic structures and functions and upregulated in AD GO terms associated with inflammation. As outlined in the attached MS, inflammation-associated proteins have recently been shown to be associated with both tau (Wes 2014) and A beta (Bhaskar 2011) - related toxicity and with signal transduction pathway disruptions that induce cell cycle re-entry. The E2- immunostaining pattern seen in Figure 2 is thus highly consistent with the early dominance of E2- tau isoforms in AD CSF (Technical progress report for Q1), the significantly higher tendency of E2- tau to be secreted in cellular models, and the pattern of exosome fraction proteins seen with E2- and E2+ tau expression in SH-SY5Y cells (Figure 3).

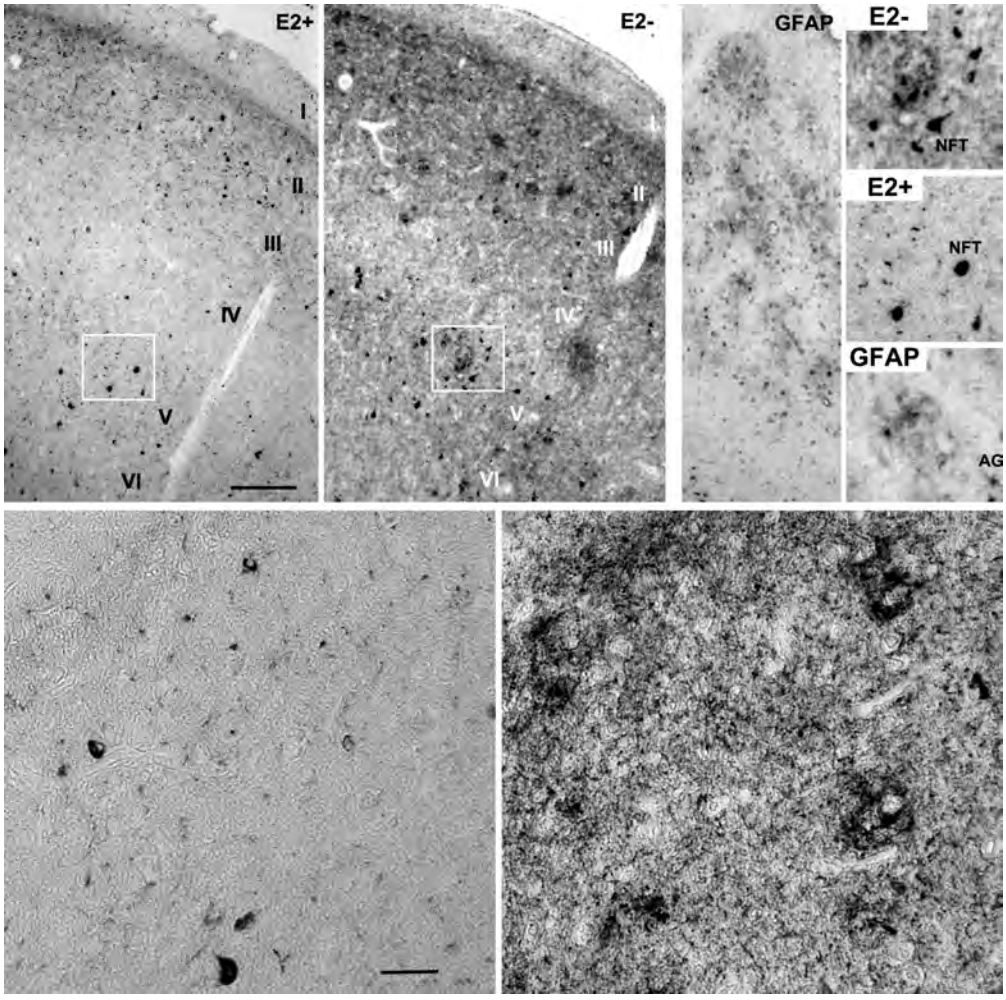

Figure 2 Example of E2- vs E2+ immunostaining in adjacent isocortical sections. Low magnification views of E2+ tau immunolabel (DC39 - top left) show strong labeling of neurofiobrillary tangles (NFTs), pre-tangles and some processes distributed through the pyramidal cell layers (2-3, 5-6) of isocortex. A higher magnification view of a different section is shown at below left. An adjacent section immunostained for E2- tau (9A1) at top center (low mag) shows much greater involvement of perimembranous structures, processes and what appear to be synaptic boutons as well as NFTs than is evident with E2+ tau. Most interestingly, E2- tau, but not E2+ tau appears to colocalize with senile plaque profiles revealed by GFAP (glial fibrillary acidic protein) immunolabel (see insets and panel at right). The patchy "plaquelike" pattern can be better seen in the high mag image at bottom right. AG - activated astroglial cell; Scale bars: 200 microns (top); 100 microns (bottom). Cortical layers are indicated by Roman numerals.

We have found similar but somewhat different patterns of E2- vs Es+ tau immunolabel in the CTE cases that we have examined; but these patterns appear to be more variable and may depend on the distance of the region being examined from lesion foci. We have recently identificatied a mAb marker (a combination of core domain and sidearm domain mAbs against neurofilament proteins) that highlights axonal damage and that we expect will be very useful in interpreting CTE pathology with respect to the E2-/E2+ pattern once we begin processing more CTE cases.

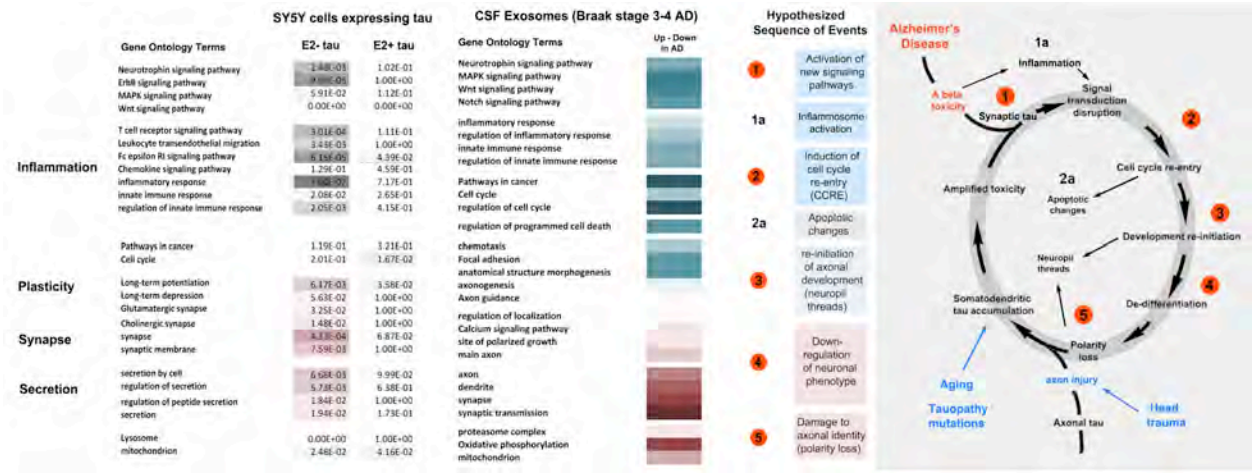

Figure 3 The pattern of protein recruitment to exosomes with the expression of E2- tau isoforms is consistent with that seen in the AD 3-4 CSF exosomal proteome and suggests that E2- tau could play a distinctive role for in AD and non-AD tauopathy pathogenesis. GO term analysis of full length and N terminal half E2- and E2+ tau isoforms. A heat map comparing the String 9.1 derived p-values for the enrichment of inflammation, synaptic and secretion-associated GO terms is given at left. Note that the signaling and inflammation proteins are not uniformly "upregulated in AD" as they are in early AD CSF; the exosomal fraction proteins from SH-SY5Y cells thus do not reflect the overall increase in "upregulated in AD" proteins seen in the CSF exosomal proteome in early AD (see attached MS). The sequence of initial cellular events that we have proposed to account for AD cytopathogenesis are shown at right (red circles) These account for the enrichment of upregulated (blue) and downregulated (pink) in the nonEVE subset of the AD 3-4 proteome. The corresponding GO terms are shown in the heat map, with the degree of enrichment returned by String 9.1 for each term is shown (center). The color reflects the difference in enrichment between up and downregulated (up p value exponent - down p value exponent) proteins for each term. The indicated sequence of events is schematized at right and can be summarized as: **(1)** Perisynaptic Abeta deposition interacts with postsynaptic tau in plastic neurons to change tau mediated signal transduction, via multiple pathways involving **(1a)** Ab-induced inflammation and the abnormal activation of kinases such as fyn and GSK3b. **(2)** This results in cell cycle re-entry, causing **(2a)** abortive apoptotic changes and **(3)** the downregulation of the differentiated neuron state. It may also result in synaptic or exosomal secretion of tau and associated proteins from dendrites. Downregulated neuron-specific proteins are shed via the exosome pathway, damaging the axodendritic polarization of the neuron and its ability to efficiently sequester tau to the axon **(4)**, resulting in amplified dendritic toxicity as more tau localizes to the dendrites and is exposed to perisynaptic A beta and enhanced toxicity **(5)**. The highly significant enrichment of "downregulated in AD" proteins seen with E2- tau constructs raise the possibility that E2- tau isoforms may be secreted preferentially via the "nonspecific" pathway (described in the attached MS) as well as via enhanced recruitment via speciofic tau-tau interactions (see Appendix: attached Excel file).

Aim 2: Biochemical interactions between Tau & the exosomal proteome

Overview:

Key results from the completed work after 1 year are as follows:

- Construction of inducible, stably transfected cell lines for all tau constructs is nearly complete for NB2As and is in progress for P19 and SH-SY5Y cells
- Technical progress on localization studies for E2-/E2+ tau isoforms in P19 cell culture models
- We have (provisionally) found 2 elements of AD pathogenesis visible in CSF exosomes that are correlated with both the early AD exosomal proteome (see attached MS) and with the overexpression of E2-, but not E2+ human tau isoforms in exosomal fractions from SH-SY5Y cell cultures. These elements are a) the activation of inflammation-associated pathways and b) the downregulation of synapse-associated genes (see Appendix: attached Excel file).

A) Proteomic association of E2+/- isoform expression in cell culture

Tau-expressing vector generation This sub Aim includes the generation of necessary constructs and cell lines necessary to localize E2+ and E2- tau in cultured neuronal cells as well as the bioinformatic analysis of proteins identified by mass spectrometry in various cellular fractions and extracellular exosomes. Some of these constructs have been (and are being) used in SH-SY5Y cells to generate the bioinformatic data described in Figure 3 and in the attached Excel file. Over the last year we have obtained of constructed vectors that express E2- and E2+ versions of various species of tau in cell culture through either constitutive or TET-On inducible plasmids. Sequencing of all of the samples described here was performed to verify starting material and primers were ordered for PCR and restriction cloning. Four tau genes (4R0N, 4R2N, E23- 1-255 and E23+ 1-255) have been spliced into the constitutive pcDNA3.1+ vector along with an N-terminal EGFP. In order to investigate the distribution, secretion and exosomal recruitment characteristics of N terminal and full length tau constructs that are known to be toxic in cell culture models, we have also constructed E2- and E2+ versions of a) the hyperaggregating 1PO/2PO/3PO family of tau mutants and b) the "calpain fragment" or toxic N terminal tau fragment (45-230) using the Flp-In T-REx system for TET-ON control. We have also added a N-terminal myc tag to all of these constructs for downstream purification and protein analysis. The expression of all of these vectors has been achieved as stably expressing constructs in NB2A cells and some of them (the constitutively expressing ones) have also been stably expressed in SH-SY5Y cells (see discussion below). We are currently expanding the set of lines available for this project by putting the basic E2-/E2+ full length and N terminal half constructs in P19 cells (see discussion under Aim 2b below).

Bioinformatic analysis of exosome fraction proteomes from E2- and E2+ -expressing SH-SY5Y cells We have completed the first phase of analysis of the exosome fraction proteome generated by the stable expression of full length (4R0N and 4R2N) and N terminal half tau constructs that either possess (E2+) or lack (E2-) the N terminal domain exons 2 and 3. The raw data for this analysis is provided in the Appendix (attached Excel file), where Fisher Test (FET) based analyses of GO term combinations are provided. The following Gene Ontology (GO) terms were used to assemble the compound categories of this analysis:

Signaling: (Neurotrophin signaling pathway, ErbB signaling pathway, MAPK signaling pathway, Wnt signaling pathway).

Inflammation: (Fc receptor signaling pathway, Leukocyte transendothelial migration, Chemokine signaling pathway, innate immune response, Inflammatory response)

AD (neuronal structures, excluding synapses): (axon, dendrite)

Synaptic plasticity: (Long-term potentiation, Long-term depression, Learning or memory, Glutamatergic synapse)
Synapse: (synapse, synaptic membrane, Synaptic transmission)

Secretion: (secretion by cell, regulation of secretion, regulation of peptide secretion, secretion)
Protein turnover: (Lysosome, mitochondrion*, Oxidative phosphorylation*)

* the rationale behind this is that mitochondria are the most characteristic targets for macroautophagy, which is diverted to exosomes in neurodegenerative disease, especially AD

The "exosome" GO term is "extracellular vesicular exosome" (EVE).

We identified a total of 2384 proteins in the exosome fraction of the cultures expressing full length E2- tau; 1891 proteins/full length E2+ tau, 1823 proteins N terminal half E2- tau, 1757 proteins N terminal half E2+ tau. 1364 proteins were identified in non tau-expressing cultures – these were removed from excluded from the E2-/E2+ list analysis.

Initial results of this analysis can be summarized as follows:

- Exosomal fraction proteins in the EVE GO term from E2- tau expressing SH-SY5Y cells are significantly more tau/Abeta-associated than are those from E2+ expressing cells.
  Both full length and N terminal constructs of E2- isoforms show a 2.5 fold greater presence in Set 1 (the top 10% of proteins by tau/Abeta links). There is no difference in the proportion of EVE and recruited (non-EVE) proteins between E2- and E2+ exosomal fractions
- The exosome fraction in SH-SY5Y cells expressing full length E2- tau is significantly enriched in "downregulated in AD" proteins in exosomes. The E2+ tau proteome is not, but the small sample size of the "unique set" used (i.e. the 100-200 proteins that were not in the controls or in the datasets for other isoforms) precludes more detailed conclusions. There is no apparent difference in the degree of tau association between E2- and E2+ proteomes.
- The pattern exhibited by the E2- assocoated proteome is broadly similar to the CSF exosomal proteome in AD, except that it hs more "downregulated in AD" proteins. It is not clear whether this is different from the E2+ pattern (sample size limitations).
  In this pattern, signaling and inflammation are the most upregulated pathways, with neuronal functions being mostly downregulated but still tau associated, and protein turnover mechanisms are least tau associated.

We are currently starting to test "exosome depleted FBS" supplements in cultures for the purpose of media exosome collection.

B) Characterization of secretion-associated protein in cell culture and tissues

P19 cell differentiation showing axon-dendrite polarization This Aim was delayed for a considerable time by our inability to get detectable axon-dendritic polarization from NB2A cells cultures during the first year of the grant. During Q4, we substituted P19 cells, a murine line used in several studies of neuronal polarization (Aronov 2001) and have since successfully generated cultures where the neuronal phenotype is sufficiently well differentiated to allow confocal colocalization studies for demonstrating physical colocalization of E2- vs E2+ associated

proteins (Figure 4), which will be initially identified for testing using bioinformatic information emerging from Aim 2a. We are currently initiating the use of Campenot chamber cultures with P19s with the intention of examining axonal vs somatodendritic exosomal secretomes.

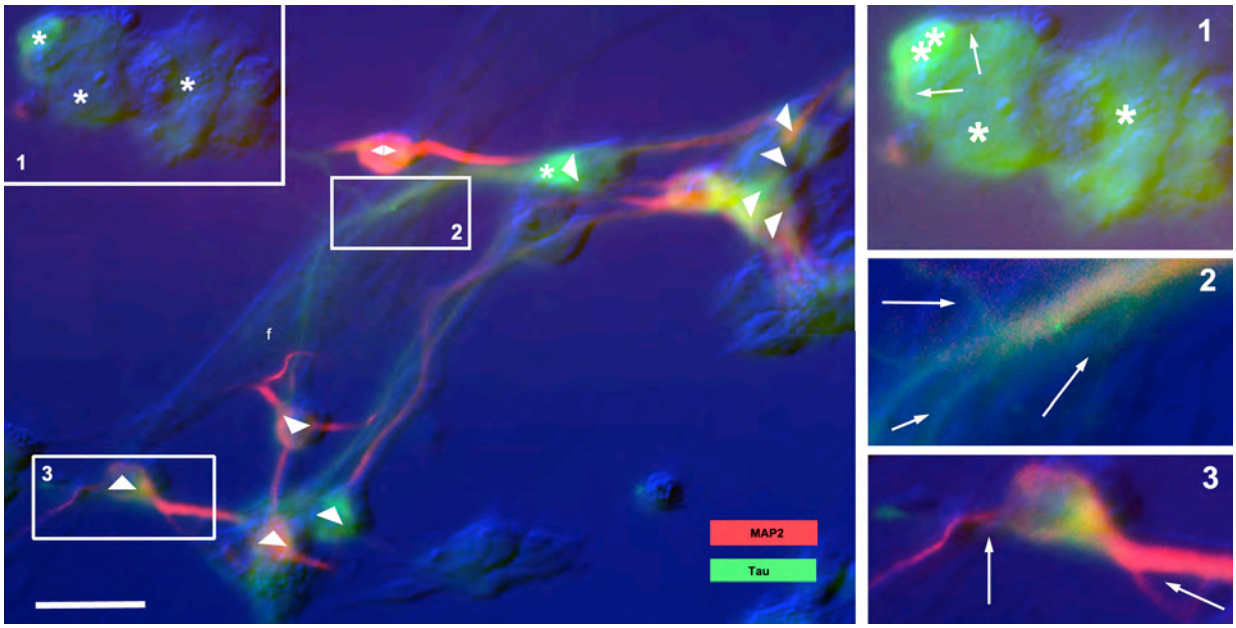

Figure 4 **Polarization of axonal and somatodendritic markers in cultured P19 cells** P19 cultures after **4 days** of differentiation in suspension culture (Petri dish) followed by **4 days** of process outgrowth in **35 mm** tissue culture plates show P19 cells in various stages of neuronal differentiation. Monoclonal Abs specific for human MAP2 (a dendritic marker - red) and human tau (an axon marker - green) illustrate stages 1 - 4 of neuronal polarization as described by Baas et. al. (1988). Insets show enhanced tau and MAP2 (insets1-2) or MAP2 (inset 2) immunolabel. Stage 1 neurons have left the cell cycle but have not elaborated neurites and show little tau expression and no MAP2 (Inset 1, single asterisks, top left). Inset 1 also shows a cell in Stage 2 (double asterisk), with minor neurites (arrows) and somewhat higher levels of tau expression. Stage 3 of neuronal polarization occurs when one process begins to elongate rapidly and forms an axon. While none of the P19 cells shown here illustrate Stage 3 well, several cells are in Stage 4, where non-axonal neurites begin to differentiate into dendrites, which become MAP2 positive. Most of the Stage 4 cells here show a multipolar phenotype (triangles) with one showing a more bipolar morphology (opposed carets). P19 cells show multiple morphometric features of dendritic development (insets 2 and 3), including a tapered morphology and the tendency to branch at acute angles (arrows). Inset 2 (early Stage 4) shows that dendritic morphology (tapering, acute branching) is established before much MAP2 has been sequestered in dendrites. Later Stage 4 cells (Inset 3) show these characteristics together with a much higher MAP2/tau ratio. Scale bar **25** microns. This micrograph was taken using the Zeiss Axio VertA fluorescence microscope funded by this grant.

Summary of Problems Encountered

Aim 1a:

- Preliminary attempts to fractionate hippocampal homogenates by centrifugation into mitochondrial/lysosomal and ER/Golgi components have been analyzed via bioinformatic profile (assembly of GO profile of enriched terms using String 9.1) and appear to be unsatisfactory (i.e. are inconsistent with nominal fraction identity).

- Also, more attention/research effort was given to the CSF bioinformatic analysis than had hitherto been expected, as this analysis proved to be difficult and required an extended learning curve on the part of the PI. The AD-associated GO functions initially chosen had to be repeatedly updated in light of new publications; notably, the effect of inflammation pathways had to be integrated into the analysis at a late date, resulting in extensive reanalyses and the updating of the attached study. This work was the main factor in the delayed submission of this report.
- New limits placed on sample size per patient available from the BU AD brain bank have delayed Western Blot and ELISA studies of non-exosomal CSF and brain homogenate fractions in favor of mass spec based identification of proteins in these fractions and their analysis using bioinformatics as described (see attached MS). This will also be used to verify fraction identity.

Aim1b

- Progress on Aim 1b has been slower than hoped owing to a) initial delays in acquiring HRPO regulatory approval for the use of human tissues b) the limited availability of CTE samples (in particular) combined with c) an extended learning curve in handling immunohistochemistry issues peculiar to heavily fixed post mortem human brain. Immunohistochemistry on human brain tissue was delayed by approximately 7 months until such time as HRPO requirements had been completed and approval to begin work was granted on 3/20/2014. Further delays toward goals are not anticipated. Localization has been consistent under multiple conditions and across several samples. In addition, there has been a variability of intensity between samples which may serve to indicate degree of neuropathy. Furthermore, improvements to histology protocols made in Q4 have greatly improved epitope visualization, eliminated background artifact, and improved tissue preservation without sacrificing throughput.

Aim 2a

- The primary delay with this Aim concerned our finding that transient transfection of NB2A and SH-SY5Y cell lines yielded results that were too inconsistent to be used for exosomal proteome generation via mass spec analysis. The consequence was that we had to generate stably expressing cell lines using standard selection protocols, which delayed progress by several months.

Aim 2b

- The failure to achieve sufficient axonodendritic polarization of NB2A cells for microscopic localization studies of Aim 2b was reported in the Q3 report and caused us to substitute P19 cells for NB2As for these studies. We have acqured the P19s and have successfully differentiated them (Figure 2). We therefore anticipate nop futher hindrance with this Aim and will be proceeding to confocal studies as originally planned.

4. Key research accomplishments

Completed analysis of Early AD CSF exosomal proteome.

Attached MS on the characterization of the CSF exosomal proteome in early AD (intended submission to Science Translational Medicine) This study will be the first systematic comparative characterization of the CSF exosomal proteomes of AD patients vs nonAD controls and will establish a number of "firsts" that are of key significance to the field of AD biomedical research. While rapid progress is being made currently on the molecular characteristics of toxic proteins in neurodegenerative disease and in particular, how these characteristics may favor interneuronal lesion spreading in disease pathogenesis, very little is yet known about the cellular context of the events that mediate tau secretion, uptake and toxicity in the course of tauopathy pathogenesis. Similarly, while progress has been achieved in increasing the resolution and precision of current biomarker-based diagnostics for AD, the fundamental nature of these diagnostics remains unchanged - they are based on the early appearance of a combination of a few biomarkers (for AD, typically tau, phosphotau, and A beta) as verified by large scale clinical studies. In this study, we have used bioinformatics to bridge the currently extensive gap between existing knowledge of tau/A beta toxicity and the cellular context of tau secretion and the molecular characteristics of the tau molecule. This study is thus both the first to attempt a "fingerprint" based AD diagnostic using exosomes and the first one to support it with a biologically comprehensive model of the cellular events leading to AD-associated secretion that is consistent with most recent literature.

We have identified other provisional results that will be of high importance/impact if verified by work during the second grant year:

Chief among these are the results of our initial analysis of the exosomal fractions of E2- and E2+ expressing SH-SY5Y cells. These suggest 2 plausible mechanisms for the preferential secretion of E2- over E2+ tau isoforms that are consistent with results emerging from other Aims of this project:

> The finding that EVE set exosomal proteins associated with E2- tau isoform expression are significantly more linked to tau/Abeta than E2+ associated exosome set proteins suggest that the "direct" recruitment pathway to exosomes may be more effective in bringing E2- tau isoforms to exosomes (see the attached MS for an account of these pathways).
>
> The finding that E2- tau is associated with significntly more "downregulated in AD" proteins than E2+ tau suggests that E2- tau isoforms and fragments may be more closely associated with tau toxicity than other isoforms, since toxicity is a hallmark of the nonspecific recruitment pathway. This might increase the recruitment of E2- tau via the "indirect" pathway as well. Moreover, this hypothesis is subject to verification by comparing E2- and E2+ versions of tau species known to be toxic in cell culture (Iliev 2006, Park 2005).

Conclusions and Future Directions

Conclusions/Impact/Military significance Overall, this project has progressed satisfactorily during the first year, with some progress in all Aims and no emergent difficulties that threaten the eventual achievement of all of the Aims as originally proposed. The general and military benefits that were described in the funded proposal thus remain valid: The project will take a major step toward determining the validity and utility of using the presence or enrichment of E2- tau isoforms as a prospective biofluid biomarker diagnostic for AD, as originally planned. In addition, it will use a new approach to generating an AD biomarker that should have significant future impact: the use of proteomic data from a well defined cellular/extracellular fraction (i.e. exosomes) that now appears to play a major role in neurodegenerative disease pathogenesis to generate a disease-specific "fingerprint". The significance of this approach is increased by the findings described in our paper (see attached MS) i.e. that we have now characterized 2 discrete routes by which nonexosomal proteins may be recruited to exosomes, one of which (the "direct" route) is AD specific and involves protein-protein interactions among proteins upregulated in AD. Moreover, these routes are consistent with a plausible and testable hypothesis of AD pathogenesis that accounts for the synergy between AD and traumatic brain injury. These features should be of great future consequence both to the military and to the public at large.

Year 2 of this project Research effort during the second year of funding will be directed at the following:

- Accumulating a sufficient sample size of adjacent and double-labeled E2- and E2+ immunostained samples from a large enough patient pool to confirm provisional findings of E2- and E2+ distribution in AD and (particularly) in injured vs non-injured regions of isocortex in CTE cases.
- Western Blot /ELISA analysis of E2+/E2- ratios in subcellular fractions isolated via centrifugation and sucrose gradient from brain (after verification by bioinformatic analysis).
- Culturing differentiated P19 cells in subdivided (Campenot) chambers and the collection and analysis of axonal vs somatodendritically derived exosomes. This will be combined with resolving technical issues associated with inducible expression of E2- and E2+ tau isoforms and the culturing of P19s in media suitable for exosome collection (i.e. exosome-free FBS). Confocal analysis of tau and immunocyto/histochemically colocalized proteins selected using the bioinformatic data generated here will allow us to complete Aim 2b.

Future directions Extending cell culture based results with E2- and E2+ N terminal half and full length constructs to tau species with known cytotoxicity in cell culture. This will require the use of the TET inducible cassettes described in the Summary under Aim 2a. We will also direct some effort toward verifying the results of the bioinformatic study of early AD CSF exosomal proteome using other approaches, since this finding is of great potential consequence to the field. We are currently looking into the possibility that "downregulated in AD" proteins cleared to exosomes via the "indirect pathway" are likely more fragmented than the "direct pathway" proteins. iff so, this would offer important confirmation of the importance and disease relevance of the study described (see attached MS). If we determine that the development of axodendritic polarization during neuronal differentiation modulates the distribution of E2- vs E2+ tau isoforms, we will extend Aim 2b to studying the effects of axotomy on the distribution and exosomal proteomes generated by expressing E2- and E2+ isoforms.

6. Publications, Abstracts and Licenses

Publications:

1. **Gendreau K, Hall GF** (2013) Tangles, toxicity, and tau secretion in AD – New approaches to a vexing problem. *Front Neurol* **4**, 160.
2. **Hall GF** (2014) Report from the Tau Front: Cantoblanco 2013 J Alzheimers Dis Parkinsonism 4, e133
3. Saman S, Lee N, Inoyo I, Jin J, Li Z, Doyle T, McKee AC, **Hall GF (2014)** Proteins Recruited to Exosomes by Tau Overexpression Implicate Novel Cellular Mechanisms Linking Tau Secretion with Alzheimer's Disease. J Alz Dis: DOI:10.3233/JAD-132135
4. **Hall GF**, Jin J, Li Z AlBattah R, Nguyen K, Viswanathan R, Munkterjal D, Lee N, Jackson B, Alvarez VE, Goldstein L, McKee AC, and Saman S. The pattern of protein recruitment to CSF exosomes yields a "fingerprint" for early AD (submission ready – appended document)

Abstract:

Hall, G.F. (2013) Systems-based analysis of proteins recruited to exosomes by 4R0N overexpression and in AD. **2013 Cantoblanco Workshop, Madrid, Spain**.

Licenses: None

7. Inventions, Patents or Licenses None

8. Reportable Outcomes None

9. Other Achievements:

a) Generation of NB2A and SH-SY5Y based cell lines expressing 4R0N and 4R2N tau constructs (as described in Project Summary).

b) Creation of exosomal proteomic databases for early AD CSF samples.

c) Sudad Saman received his Ph.D. in Biology and Biomedical Engineering on the strength of work he performed towards the Aims of thes grant while being supported by it.

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11. Appendices

Submission ready MS (PDF file: AIS-MAPT STM format v52)

Supplementry information for Hall 2014 Annual Report (Excel file)

The pattern of protein recruitment to CSF exosomes yields a "fingerprint" for early AD

Garth F. Hall[1], Jun Jin[1], Zhihan Li[1], Raid AlBattah[1,2], Katherine Nguyen[1], Ramya Viswanathan[1], Dolgormaa Munkterjal[1], Norman C.Y. Lee[3], Bruce Jackson[2], Victor E. Alvarez [3], Lee Goldstein[4], Ann C. McKee[4] and Sudad Saman[1,2]

[1]Department of Biological Sciences, University of Massachusetts Lowell, 198 Riverside Street Lowell MA, USA.

[2]Mass Bay Community College Science Department STEM Division 50 Oakland Street Wellesley Hills, MA 02481

[3]Boston University Chemical Instrumentation Center, Department of Chemistry, Boston, MA USA 02215

[4]Boston University School of Medicine, Departments of Neurology and Pathology, Boston, MA USA 02215.

Correspondence to Garth F. Hall, Department of Biological Sciences,
University of Massachusetts Lowell198 Riverside Street Lowell MA, USA.

Telephone: 978-934-2893,
FAX 978 934 23044
E-mail: Garth_Hall@uml.edu

Keywords: exosome, tau-associated secretion, early AD CSF, proteomic analysis, AD neuropathogenesis, AD diagnosis

Abbreviations:

AD, Alzheimer's Disease;	CNS, central nervous system;
NDD. neurodegenerative disease	APP, amyloid precursor protein
NFT, neurofibrillary tangle	Aβ, beta amyloid peptide;
GO, gene ontology	EVE, extracellular vesicular exosome GO term;
MT, microtubule;	CSF, cerebrospinal fluid,
MID, multi-infarct dementia;	VaD, vascular dementia;
CCSF, MID/VaD control CSF;	AD 3-4, early AD CSF;
CCRE, cell cycle re-entry;	KB, Knowledge Base;
FET, Fisher's Exact test;	CST, Chi square test;

8 Figures
101 References

Abstract	237
Text	5826
Figure Legends	2070
Total	8138

It has recently been shown that aggregation-prone proteins that play central roles in the cytopathogenesis and interneuronal lesion spreading in AD and other NDDs are secreted via exosomes and that the overexpression of one of these (tau protein) can cause substantial changes in the exosomal proteome. These findings suggest that understanding the cellular mechanisms that recruit (normally) non-exosomal proteins to the exosome pathway may lead to the identification of a disease-specific "fingerprint" prognostic from CSF and/or more accessible body fluids. Here we present a bioinformatic analysis of exosomal fraction CSF proteins from 7 neurologically staged (Braak stages 3-4) clinical AD patients in comparison with 7 age matched ischemia-associated dementia (VaD, MID) cases with low (0-3) hippocampal Braak scores. Analysis of AD and control proteomes with respect to a) expression levels in AD, b) membership in the exosome GO term marker set, c) degree of physical interaction with tau/Aβ and d) measures of interproteomic connectivity suggests that proteins are recruited to CSF exosomes via 2 distinct routes. We find that the combined exosomal CSF proteome of the AD samples exhibits a coherent, AD-specific pattern that can be correlated with key events in AD cytopathogenesis. These results suggest that a "fingerprint" based on the exosomal proteome may exist that might be detectable in the CSF and more peripheral body fluids at or prior to the earliest stages of AD, with important implications for both AD diagnostic and therapeutic development.

Introduction The identification of neurodegeneration-associated proteins in extracellular microvesicles (exosomes) (1-5) have recently suggested that a common feature of such proteins – their tendency to form toxic oligomers and aggregates - play a significant role in both their toxicity and the spreading of neurofibrillary lesions characteristic of these diseases between neurons. This is particularly true of Aβ and tau protein, whose interaction is now generally regarded as the central etiological event in the evolution of neurotoxicity in AD. While much attention has been given to specific aspects of Aβ and tau misprocessing with respect to lesion spreading mechanisms, very little is yet known about how the cellular mechanisms that link tau-mediated Aβ toxicity to hallmark AD features such as senile plaque, neurofibrillary tangle (NFT) formation and the generation of elevated CSF tau. Nor do we understand whether and how Aβ and tau induce the disparate cellular changes that are collectively characteristic of AD (e.g. synaptic damage, cell cycle re-entry (CCRE – 6), inflammation (7) and neuropil thread formation (8-9). However, it has become increasingly apparent that such links exist; synaptotoxic changes associated with localized Aβ (10-12), perisynaptic tau (13-15) or via their interaction (16-17) are very early events in AD cytopathogenesis that appear to drive other aspects of neuronal dysfunction in AD (18-19), including tau secretion (20) excitotoxicity (21) and exosomal neurotransmitter release (22).

The findings that tau can be released to the CSF at least partly via the exosome pathway (3) and that tau overexpression in neuroblastoma cultures results in the recruitment of AD-associated proteins to secreted exosomes (23) suggest that disease-specific changes in synaptic function, vesicle trafficking and signal transduction prior to and during the onset of AD might be reflected by changes in the exosomal proteome. Moreover, since tau can be secreted in exosomes in early AD (3), and since elevated CSF-tau levels significantly anticipate neurodegeneration in AD, it seems likely that passive release from dead neurons is not an important source of CSF-tau in early AD (3, 24). Instead, misprocessing of tau and Aβ may cause them and associated proteins to be abnormally diverted to the exosome pathway in early AD (25-28) and thus provide a window on the initial stages of AD cytopathogenesis and potential AD-specific biomarkers.

Exosomes now appear to have a very broad range of functions that include cell-cell communication via microRNA and protein transfer (29-33), and may be an important route for tumor metastasis and in viral infectivity (34-35) as well as neuronal development (36-37) and NDD (38-41). The finding that protein oligomerization may cause diversion to the exosomal pathway (26) suggests that exosomes may also be a route for lesion spreading in neurodegenerative disease. While the range of mechanisms that normally recruit proteins to exosomes is still poorly understood, it now appears that changes in the contents of

exosomes may reflect either direct changes via specific protein-protein interactions and cellular events (20-24, 26-27) or indirect consequences of changes in cellular turnover mechanisms (23, 31, 42).

In this study, we characterized and compared CSF exosomal proteomes from patients with early (Braak Stage 3-4) AD and ischemia-associated non AD dementias (MID and VaD). We show that proteins not typically found in exosomes (i.e. that have been abnormally recruited to exosomes by dementia pathogenesis) differ characteristically in these two groups. We find that recruitment to exosomes in early AD results in 2 discrete protein populations distinguished by a) their status as up/down regulated in AD, b) their degree of interconnectedness based on known physical interactions and c) their degree of association with tau protein based on physical interactions. Based on these findings, we propose a comprehensive model of the cellular mechanisms that accounts for many of the characteristic neuronal changes seen in early AD.

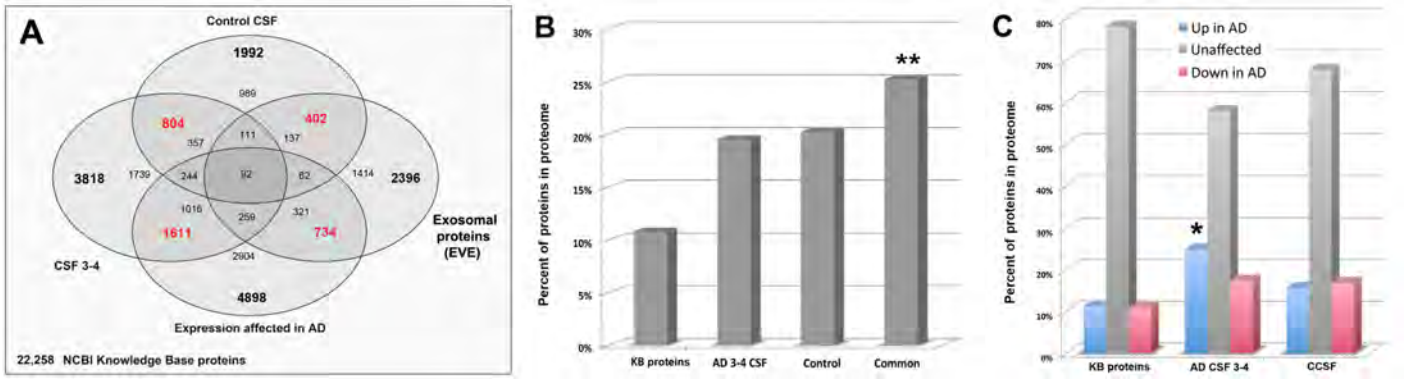

Figure 1 **Initial analysis of the CSF exosomal proteome** from postmortem incipient and early Alzheimer's Disease patients (based on clinical AD diagnosis and neurological Braak Stage 3-4 status) vs VaD/MID controls. **Panel A:** Venn diagram illustrating the size and overlap of control, early AD (3-4 CSF) and VaD/MID control exosomal proteomes according to their status as upregulated or downregulated (affected in AD) in the published datasets of Blalock et. al. (44-45) and Antonell et. al (43) and b) members of the "extracellular vesicular exosome" (EVE) GO term. Common proteins between the CSF, EVE and control CSF datasets are also identified. The total size of each of the 4 reference proteomes is shown in bolded black; the total number of shared proteins between each of them is in bolded red. The number of proteins in each discrete region of the diagram is given smaller numbers in black. Up and downregulated proteins in AD are given as a single number (expression affected in AD) and the degree of physical association with tau is not shown. **Panel B:** Both control (CCSF) and AD exosomal proteomes are enriched in EVE list proteins relative to the entire KnowledgeBase dataset, and Proteins common to both CCSF and AD 3-4 proteomes are significantly enriched over both CCSF and AD 3-4 proteomes (**, $p < 0.001$, Chi Square Test, Yates correction) **Panel C:** A significant majority of the "affected in AD" proteins were upregulated in early AD (blue bar with asterisk, $p < 0.1$, Chi Square Test, Yates correction), even though the number in the parent Up in AD and Down in AD sets was roughly equal. There was no significant difference between the proportions of up/down regulated proteins in AD in the CCSF proteome relative to the Knowledge Base set as a whole.

Results

EVE list proteins in AD 3-4 and CCSF exosomal proteomes We identified a total of 3818 different proteins in CSF samples from 7 Braak Stage 3 and Braak Stage 4 patients with clinical AD (AD 3-4), and 1992 from the 7 MID and VaD samples that served as demented controls (CCSF). There were 804 proteins that appeared in both AD and demented control samples. This common set was significantly enriched in members of the EVE GO term (*GO:0043230*) relative to both the AD ($p < 0.001$) and control ($p < 0.03$) groups (Figure 1B). Given that both sets were both derived from samples subjected to exosomal purification, this result was expected. However, when we compared the subsets of AD 3-4 and CCSF proteins detected in multiple patients, we found that enrichment in EVE list proteins was only seen in the set of 813 proteins that occurred in multiple AD patients, whereas the percentage of EVE list proteins in the set of multiple patient CCSF proteins actually decreased, resulting in a significant difference ($p < 0.01$) between the AD 3-4 and CCSF multiple patient sets (Figure 1C). All of the exosomal proteomes examined were significantly enriched in EVE list members relative to the entire KnowledgeBase (KB) (46) set (Figure 1B). However, the AD 3-4 set showed a significant admixture of self-interactive, non-EVE proteins that was not present in either the CCSF set or the KB set as a whole (Supplemental Figure 1). These non-EVE proteins were particularly prominent among the proteins that were either upregulated or unaffected by AD, as defined below.

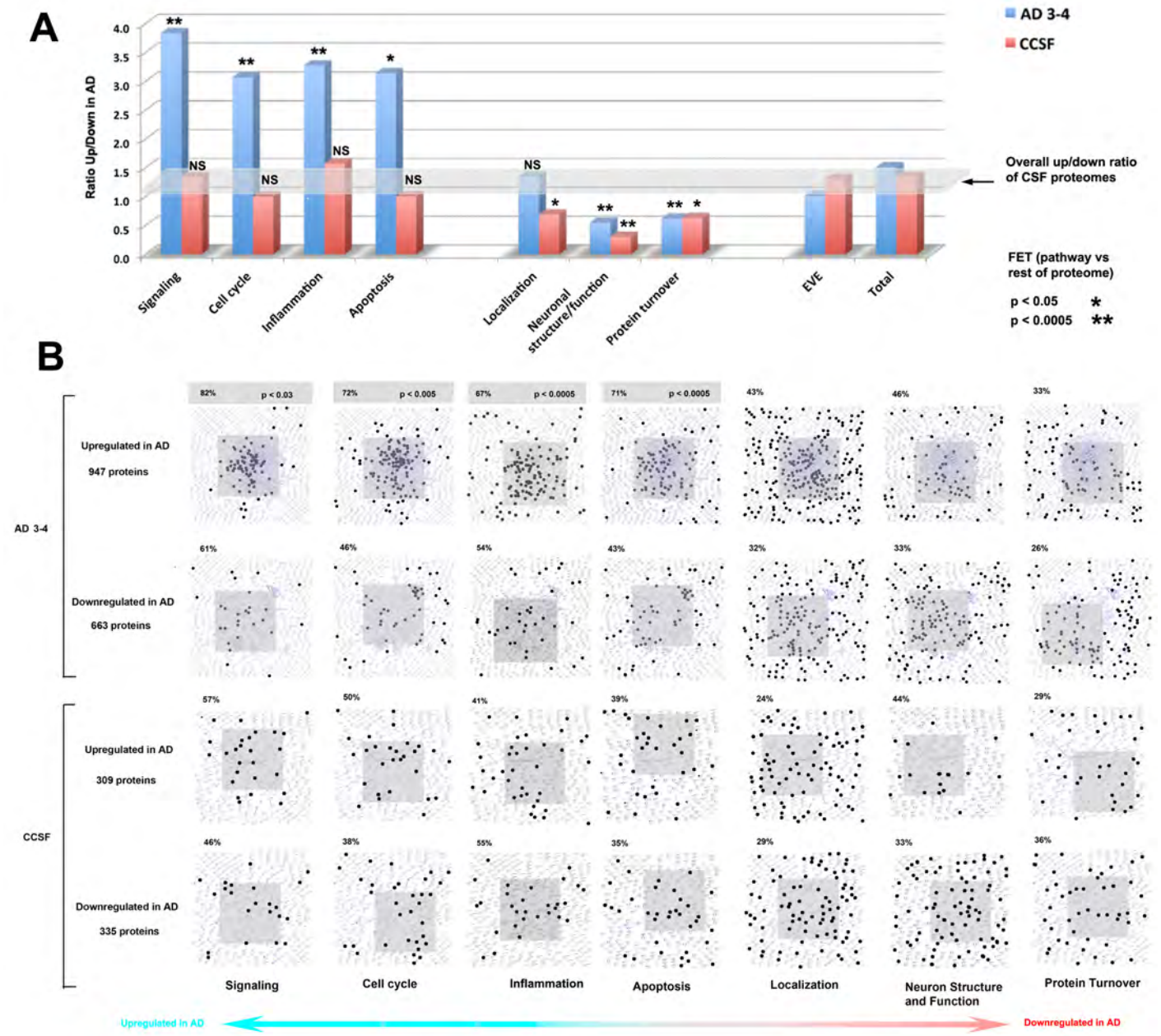

Figure 2 **Connectivity and juxtaposition analysis** shows specific enrichment and colocalization for signaling, cell cycle, inflammation and apoptosis-associated proteins among upregulated in AD proteins in the early AD exosomal proteome **Panel A:** Pathway-specific enrichment of "upregulated in AD" proteins in CSF exosomes. Results are from AD-3-4 and CCSF proteins expressed as a ratio between those upregulated vs downregulated in AD. P values are relative to the relevant CSF proteome; only physically interacting proteins are shown. Significant enrichment was seen in signaling pathways (MAPK, Wnt, ErbB, Notch, neurotrophin), cell cycle regulation (pathways in cancer, cell cycle and regulation of cell cycle), inflammation (inflammation, regulation of inflammation innate immune response, regulation of innate immune response) and apoptosis (regulation of programmed cell death). Significant enrichment of "downregulated in AD" proteins associated with neuronal structure/function (axon, dendrite synapse synaptic transmission) and protein turnover (mitochondrion and lysosome) was also seen, but was not significantly stronger in AD 3-4 than in the MID/VaD (CCSF) samples. **Panel B:** Specific differences in icon juxtaposition (i.e. clumping of physically interacting and functionally related proteins) in AD 3-4 vs demented controls (CCSF) were seen in the signaling, cell cycle, inflammation and apoptosis pathways defined in Panel 1. These pathways also showed significantly higher connectivity values vs those of the EVE term (Supplemental figure 5). All pathways, functions and structures that were not upregulated in AD (e.g. localization), or that were significantly downregulated (e.g. neuronal structure and function and protein turnover show no significant differences between AD and controls in either icon juxtaposition or connectivity and are very close to the baseline percent coverage expected for random icon distribution.

Signaling and cell cycle associated proteins that are upregulated in AD are selectively enriched in CSF exosomes We used supplemental lists of upregulated and downregulated proteins from late onset and familial AD from studies by Blalock (44-45) and Antonell (43) respectively to generate a baseline for our systems-based analysis of early AD and demented control exosomal proteomes. A complete tabular description of this analysis is provided in Supplemental File 2. Analysis of the background protein set (KB) showed that several signaling pathways (Neurotrophin *hsa04722*, MAPK *hsa04010*, Wnt *hsa04310*, Notch

hsa04330, ErbB *hsa04012*) and cell cycle regulation GO terms (Cell cycle *hsa04110*, regulation of cell cycle *GO:0051726*) were strongly upregulated in AD, whereas GO terms for neuronal structures and functions (axon *GO:0030424*, dendrite *GO:0030425*, synapse *GO:0045202*, synaptic transmission *GO:0007268*) and protein turnover (mitochondrion *GO:0005739*, lysosome *GO:0005764*, Oxidative phosphorylation *hsa00190*) were strongly downregulated (Supplemental File 2). Comparative analysis of these pathways in the AD 3-4 and CCSF sets using the entire KB proteome was hindered by the very large number of poorly characterized proteins in both the entire KB set (22,258 proteins) and the AD 3-4 and CCSF exosomal proteomes. Since Aβ/tau interactions are the central and defining cellular event in AD relative to other neurodegenerative syndromes (47-49), we performed a parallel analysis in which only the top 30% of KB proteins (a total of 6376) based on the extent of their known direct or indirect physical interactions with Aβ and tau. The pathways chosen for this analysis were either known to be involved in AD cytopathogenesis (i.e. signal transduction, control of cell cycle, apoptosis) and/or known to involve APP or tau (neuronal polarity, neuronal structure and function). Each of these was represented by composites consisting of the union of members of relevant GO terms (listed in their entirety with GO IDs in Supplemental File 2). A separate analysis of the excluded proteins was also performed showing no significant correlations between protein expression in AD and the above pathways. A complete description of both of these analyses is given in Supplemental File 2.

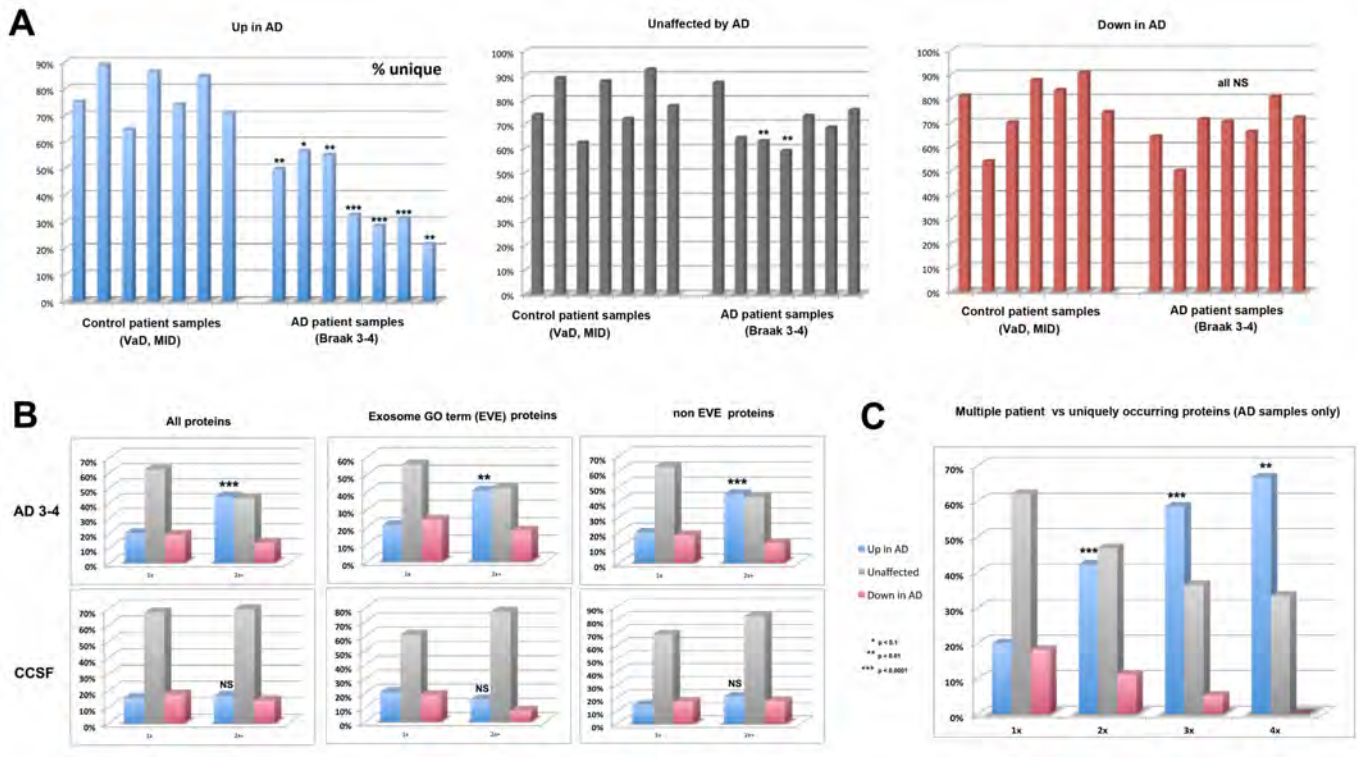

Figure 3 Proteins that are "upregulated in AD" are more consistently identified in AD 3-4 CSF samples than in MID/VaD (CCSF) controls **Panel A** shows the percentage of proteins uniquely identified in each of the CCSF (left bars) and AD patients (right bars) of each of the graphs shown. Among the proteins upregulated in AD (23, 43-45), the percentage of unique proteins identified was lower in each of the AD patients than in any of the demented controls, and was significantly ($p < 0.05$) lower than the mean control value in all but 1 (single asterisk) of the 7 AD patients. By contrast, none of the patients showed a significant difference between AD 3-4 and CCSF patients in the proportion of downregulated proteins present. **Panel B**: Both exosome list (EVE) and non-exosome (nonEVE list; i.e. recruited) proteins contribute to the enrichment in "upregulated in AD" relative to "downregulated in AD" proteins in the AD 3-4 exosomal proteome (***, **), whereas neither EVE list nor non-EVE list proteins in the controls showed significant disproportion between up and downregulated in AD proteins (NS). **Panel C:** AD-associated changes in gene expression are well correlated with the consistency with which individual proteins were identified in CSF exosomes. "Up in AD" proteins were selectively enriched in AD 3-4 relative to CCRF in multiple patients, with the degree of enrichment increasing as a function of the number of patients in which the protein was identified, suggesting that protein-specific interactions mediate their recruitment to exosomes.

Direct comparison of the AD 3-4 and CCSF datasets (both with and without the exclusion of the bottom 70% Aβ/tau subset) showed a significant enrichment of "upregulated in AD" proteins in the AD 3-4 CSF proteome that was not present in the CCSF proteome (Chi Square test, Yates correction) even though roughly as many upregulated (2492) as downregulated (2406) proteins were identified by the Blalock and

Antonell studies as being affected by AD pathogenesis. The enrichment of AD 3-4 CSF exosomes in pathways upregulated in AD was also significant relative to that seen in the KB set (Supplemental Figure 1).

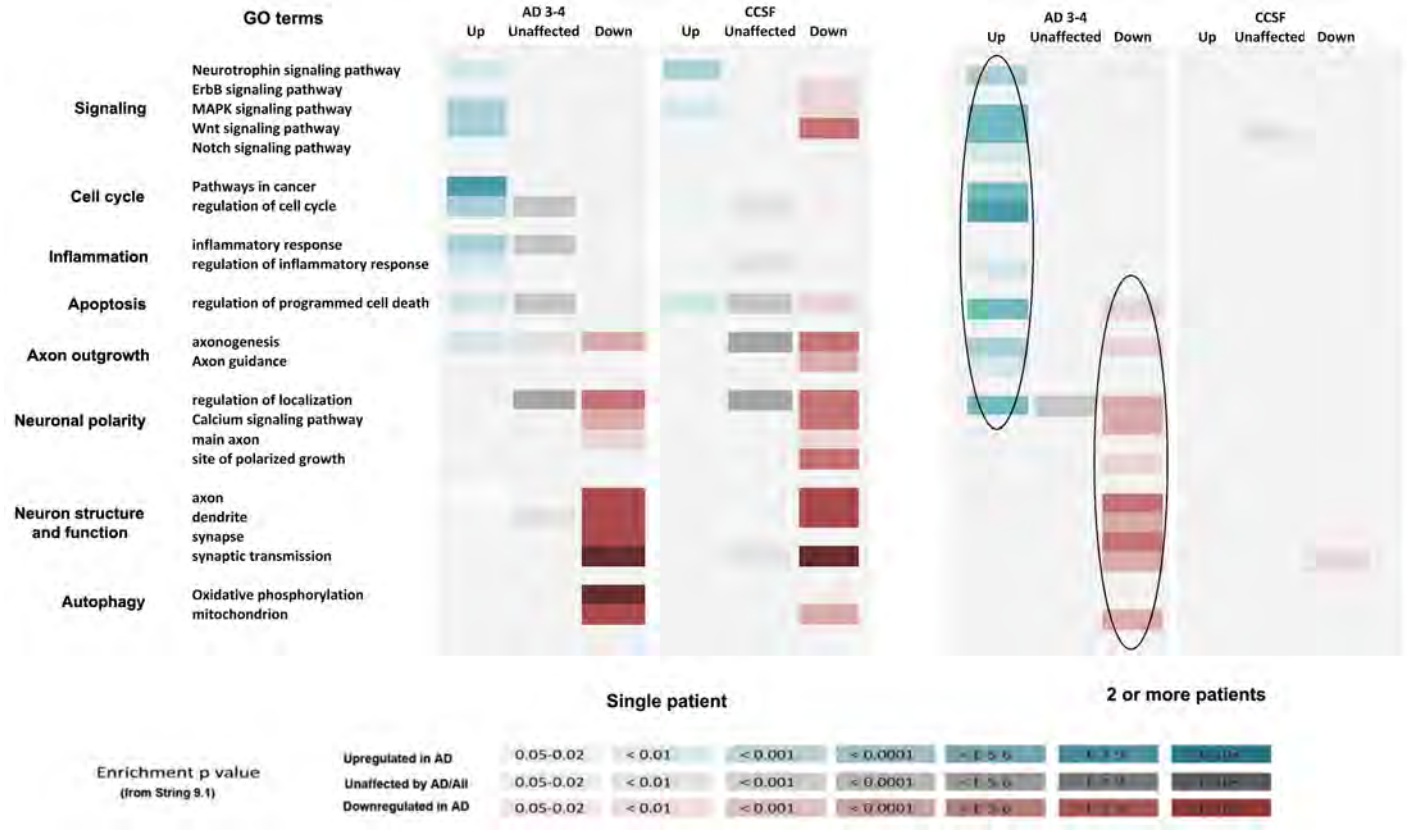

Figure 4 AD-specific effects on specific GO terms are seen primarily in the multipatient proteome A heat map showing the enrichment of uniquely occurring proteins (left) and multipatient proteins (right) in the AD and CCSF exosomal proteomes for GO terms defining functions and pathways related to signal transduction, cell cycle, axonal development, neuronal polarity and features of differentiated neurons. The degree of enrichment in exosomes returned by String is shown as the p value for each GO term, which is intensity coded for upregulated (blue) unaffected (gray) and downregulated (red) proteins in each pathway according to the key at the bottom of the figure. Although AD-specific changes could be distinguished from VaD/MID associated change using pooled GO terms defining some specific pathways (see text), a clear AD–specific "fingerprint" only emerges when the analysis is limited to proteins that occurred in multiple AD 3-4 or CCSF samples (right panels). In the multipatient proteome, there was a clear and specific enrichment of "upregulated in AD" proteins involved in signaling, axonal development and the regulation of cell cycle and apoptosis, and of "downregulated in AD" proteins involved in neuron-specific structures and functions in AD 3-4 (oval outlines at right) that were not apparent in the CCSF multipatient proteome.

String connectivity maps based on physical interactions alone (Figure 2) showed a much greater degree of interaction in the AD set than was present in either demented (i.e. MID/VaD) controls or an online control CSF exosome set (50). This was largely due to interactions between proteins that were upregulated in AD and were not present on the EVE GO term list. Nearly half (406) of the 819 non-EVE proteins present in the AD CSF proteome that were listed as being upregulated in AD showed physical interactions at moderate confidence (0.4, String 9.1). This was significantly greater than the physical interactivity of non-EVE proteins listed as being downregulated in AD ($p = 0.0001$, CST) (23, 43-45). A similar but smaller connectivity difference was seen with EVE list proteins ($p = 0.002$, FET). By contrast, there was no significant increase in the connectivity of upregulated vs downregulated in AD non-EVE proteins from the VaD/MID control subjects ($p = .2336$, FET). String-based connectivity maps (Figure 2) show that networks of increased physical interaction were present for specific cellular functions associated with upregulated proteins in AD, including several signal transduction pathways (Wnt, ErbB, neurotrophin, MAPK, Notch) and the regulation of the cell cycle. Other cellular functions associated with tauopathy-linked events (e.g. spliceosome, RNA splicing) that were not significantly upregulated were not analyzed further. Physical interactivity based on the connectivity scores between individual proteins was significantly increased for signal transduction (*GO:0007165*, $p < 0.0001$ FET), positive regulation of cell cycle (*GO:0045787*, $p < 0.001$ FET) and pathways in cancer (*hsa05200*, $p < 0.0001$ FET) relative to the EVE list protein set (i.e. the 2396 proteins recognized by String 9.1). These terms were also significant relative to the VaD/MID demented control proteome in terms of the degree of icon juxtaposition (clumping) exhibited relative to the EVE

proteome (Figure 2). Also, general signal transduction and cell cycle regulation GO terms were enriched in the EVE proteome as a whole, suggesting that the significant differences seen in these functions between AD and MID/VaD control proteomes may be due to differences in the mechanism by which non-EVE proteins are recruited to exosomes.

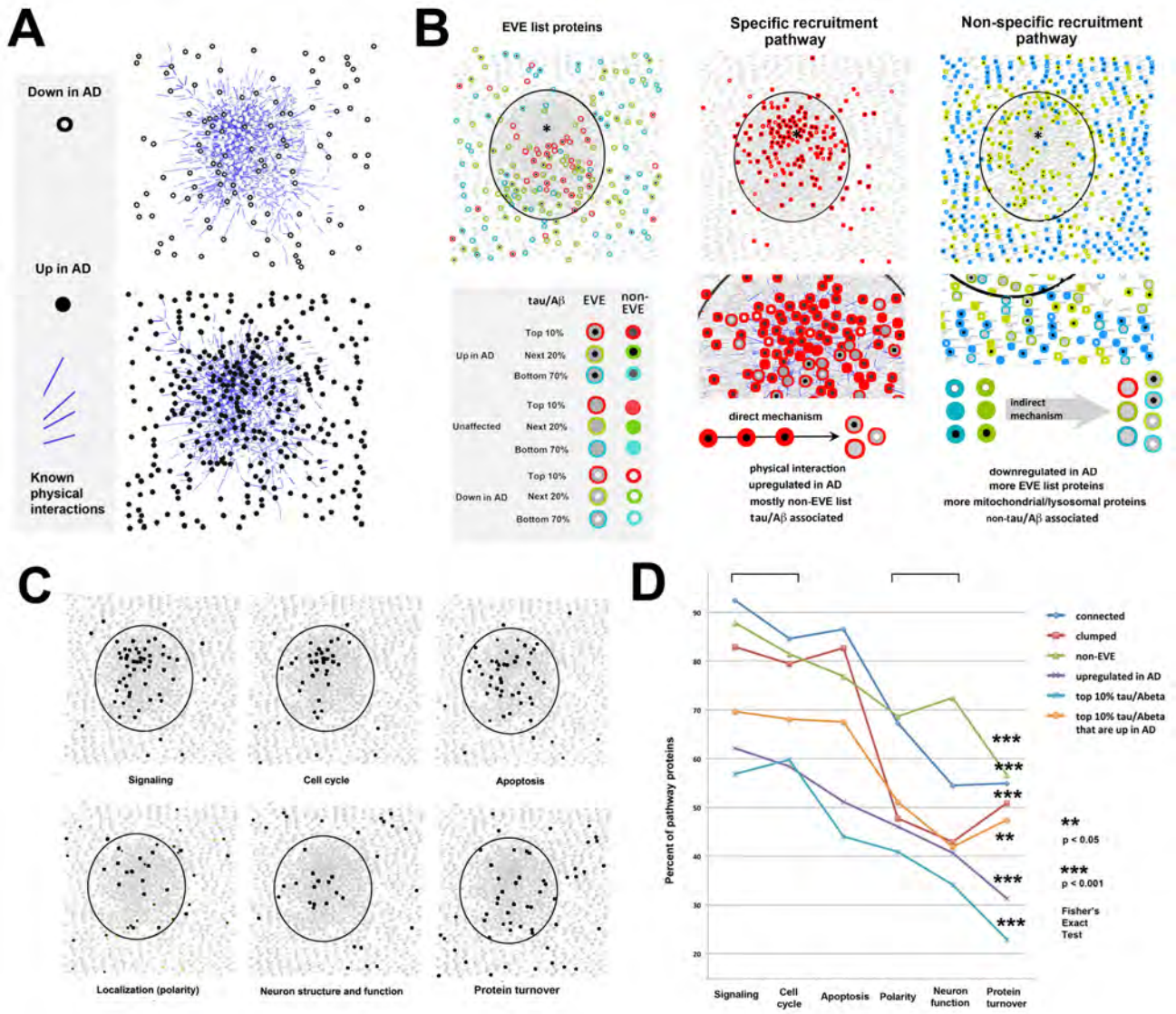

Figure 5 Multivariate analysis of the early AD multipatient proteome Analysis of the AD 3-4 multiple patient proteome suggests that normally non-exosomal proteins are recruited to exosomes via 2 distinct mechanisms in early (Braak 3-4) AD depending on whether they are upregulated or downregulated in the course of the disease. **Panel A:** String connectivity maps (physical interactions only, moderate confidence) showing physical interactions at moderate confidence (0.4 on String 9.1) among downregulated (top) and upregulated (bottom). Proteins downregulated in AD do not colocalize and are much less physically interconnected than upregulated (bottom, ring). **Panel B:** EVE list proteins (left) were not themselves differentially "clumped" (i.e. inside the ring) relative to other proteins, but upregulated that are associated with APP/tau (i.e. all red icons) are. Insets show 2 patterns of associated between recruited and EVE proteins. Upregulated in AD, tau/Ab associated proteins that interact directly (inset 1) involve a lower proportion of EVE proteins than do other proteins, especially those downregulated in AD (inset 2). **Panel C:** String diagrams illustrate cell function-specific differences in physical connectivity associated with the 2 recruitment patterns illustrated in A and B. Proteins in the "specific" recruitment pathway dominate the signaling pathways (Wnt, MAPK, ErbB, Notch, neurotrophin) and show a high degree of physical interaction. Similar patterns can be seen with cell cycle and apoptosis GO terms (top row). involved in synaptic changes in early AD associated with Aβ/tau interactions. By contrast, proteins identified by neuron-specific specializations (i.e. axon, dendrite, synapse, synaptic transmission) were significantly less interactive physically with one another and yielded String diagrams resembling the pattern seen with protein turnover functions (autophagy, mitochondrial and lysosomal GO terms) that might mediate the indirect recruitment of "downregulated in AD" proteins to the exosome pathway. **Panel D:** Quantitation of data presented in A-C. The proteins specified by the functions listed in C were divided according to whether they exhibited (from top) physical links to other proteins, icon colocalization, were members of the EVE GO term list, or were upregulated in AD and/or associated with tau or Aβ. Fisher tests between signaling/cell cycle and localization/neuronal function categories are shown (brackets).

A prominent feature emerging from the comparison between AD and control datasets was that the "upregulated in AD" portion of the AD 3-4 exosomal proteome is much more enriched in "multi-patient" proteins than the control set both on a per protein basis overall ($p << 0.0001$ CST, Yates correction) and in the proportion of proteins occurring in 3 or more patients ($p < 0.03$, CST, Yates correction). There were over

three times as many proteins from the AD samples that were identified in 2 or more patients relative to MID/VaD controls (813 vs 248). Most of these “multi-patient” proteins (96.3% and 84.6% respectively) were not shared by the AD 3-4 and CCSF protein sets. Moreover, a significantly larger proportion of those multipatient proteins that occurred in 3 or more patients were present in the AD 3-4 than the CCSF set (148 vs 24, $p < 0.02$, FET). Such “multiple patient” proteins were selectively enriched in the “upregulated in AD” subset of AD 3-4 relative to the MID/VaD samples (Figure 3). This pattern was highly significant (CST $p << 0.0001$) and also highly consistent among the 7 AD samples analyzed, with 6 of the AD samples showing significantly fewer ($p < 0.05$) “unique” proteins than the CCSF set overall, and all having fewer “multiple patient” proteins than any of the control samples examined (Figure 3a).

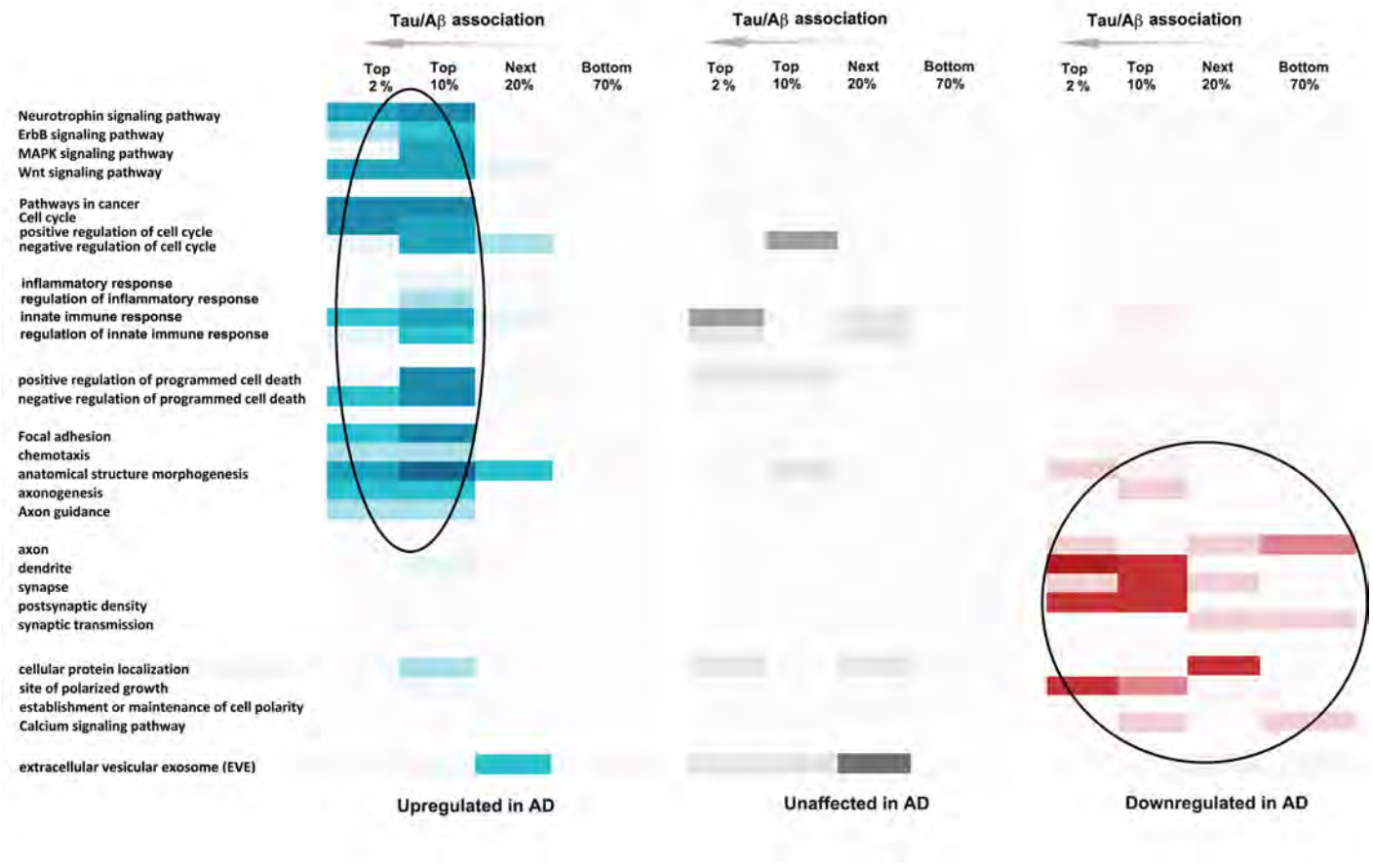

Figure 6 The degree of physical interaction with tau/Aβ correlates with pathways that are upregulated in AD in the multipatient AD 3-4 proteome Heat map showing correlations between the degree of physical association with tau/Aβ and “upregulated”, unaffected and “downregulated” proteins. Panels reflect the degree of p value enrichment for the GO terms listed as described in Figure 4. For each term, the significance of enrichment with GO term member proteins based on their degree of physical association with tau/Aβ is shown. The leftmost column of each panel shows enrichment with proteins in the top 2% (404 members); left center: the top 10% (2200 members), right center: the next 20% (4400 members), extreme right: the remaining 70% (15,659 members) of the 22,259 KnowledgeBase list proteins used in this study. More detailed definitions of these sets are given in Methods. Note that proteins that were seen to be upregulated in AD (23, 43-45) are much more tau associated (circled, blue asterisk) than downregulated proteins (circled at lower right) suggesting that interactions with tau may play a more direct role in signaling, cell cycle control and axonogenesis than they do with the quintessentially neuronal structures and functions that are downregulated in AD. Most of the “EVE list” proteins (circled at bottom) fall into the “unaffected in AD” category.

GO profile analysis of AD 3-4 vs CCSF A heat map showing the enrichment significance values calculated by String for a panel of the GO terms illustrating key cellular functions known to be affected in AD is shown in Figure 4. The AD 3-4 and CCSF datasets were separated into uniquely occurring proteins only (left panels) and proteins occurring in multiple patients (right panels). There were relatively few overriding differences evident between the AD 3-4 and CCSF sets of uniquely occurring proteins, especially among downregulated functions. By contrast, the GO term profiles of both up and downregulated functions from the AD 3-4 set of multiply-occurring proteins were characteristically different from those seen in the MID/VaD control sample (Figure 4, right panels). We found that the “upregulated in AD” subset of the 813 proteins seen in multiple AD patients were significantly associated with GO terms for signal transduction pathways and the regulation of cell cycle and apoptosis, while downregulated proteins were largely those with neuron-

specific structures and functions, including terms associated with neuronal polarization. This was very different from the profile shown by the control multipatient dataset (Figure 4, right), which showed no consistent enrichment for any of the expression subsets.

Multivariate analysis of the AD 3-4 multiple patient CSF exosomal proteome The finding that proteins common to multiple patients occur significantly more frequently in early AD than in controls and have a characteristic functional profile suggests that a distinctive set of non EVE list proteins may be recruited to exosomes by changes in specific protein-protein interactions associated with exosome secretion in early AD. In order to determine if disease-specific interactions between proteins normally present in and absent from exosomes can account for this observation, we characterized the functions and physical interactions between the 813 members of the multi-patient AD 3-4 proteome in terms of multiple variables. Three different parameters were mapped onto a common String connectivity map (physical interactions only, moderate confidence): a) status as an "exosomal" (EVE list) protein, b) effect of AD on expression and c) the degree to which each protein is associated with tau and APP, two proteins whose interaction is responsible for much (if not all) of the cascade of events underlying AD at both the cellular (47) and systemic (48-49) levels of analysis. Correlations between these parameters and the summary of current curated experimental information on protein-protein physical interactions and the degree of icon colocalization (51) provided by the String diagram were then examined as shown in Figure 5. A more detailed correlation between the GO profile of this proteome and APP/tau association is given in Figure 6.

The degree of physical interaction of a protein with tau and Aβ (APP was used in String 9.1) appears to be highly correlated with the distribution of non-EVE (i.e. recruited) proteins that are relatively closely associated (i.e. have a low number of degrees of separation) with tau and Aβ. The distribution of downregulated proteins appears to be entirely random with respect to the highly connected (mostly upregulated in AD) proteins at the center of the String maps. Interestingly, the connected proteins in the "exosomal" subsets are well connected with the upregulated "recruited" proteins (Figure 5a, bottom), consistent with a collective role for these proteins in the direct recruitment of upregulated in AD proteins to exosomes via known physical interactions. By contrast, the (mostly downregulated in AD) proteins associated with neuronal structure/function and protein localization in the multipatient AD proteome had a significantly higher proportion of EVE proteins than did the signaling pathway/cell cycle proteins described above. Enrichment for the mitochondrial and lysosomal GO terms that we used to represent protein turnover mechanisms showed a similar pattern (Figure 5c, d). Analysis of the enrichment pattern of individual GO terms (using String 9.1) in the AD multipatient proteome clearly illustrates the functional division of recruited proteins into what can be called a "specific" recruitment pathway (upregulated, physical interaction based, tau/Aβ associated) pathway and a more indirect "non-specific" pathway that primarily involves proteins associated with downregulated structures and functions in AD (Figure 5b). This second pathway is less dependent on specific protein-protein interactions and the degree of physical association with tau and APP as can be seen in both Figures 5b and 6.

Non-extracellular vesicle associated proteins in the multi-patient AD 3-4 dataset show evidence of belonging to both specific and nonspecific recruitment pathways Finally, the intense recent interest in exosome-mediated transfer mechanisms has created some confusion as to the definition of "exosome" (e.g. 52) and has led many investgators to abandon this term in favor of the more general "extracellular vesicle". In order to test the robustness of the results described above, we sought to determine whether the key features of both the "specific" and "non-specific" recruitment pathways were still present when all proteins that have been reported in extracellular vesicles were excluded. We used a compendium of proteins from Vesiclepedia (53), which lists nearly 15,000 unique proteins, to define a highly restrictive definition of "non extracellular vesicle proteins" which was met by a total of 123 members of the AD 3-4 multipatient proteome. A heat map comparing the non-Vesiclepedia/non EVE subsets of the AD 3-4, CCSF multipatient AD 3-4 and of a recently published non-demented control CSF exosomal proteome (50) is presented in Supplemental Figure 5, and the results summarized below. We found that over 75% of the proteins in both the CCSF and AD 3-4 "exosomal" proteomes (74.8 and 78.0%, respectively) were cited at least once in the Vesiclepedia "extracellular vesicle" list, compared with 87% of the nondemented control exosomal fraction (50). This last set is almost identical to the multipatient AD 3-4 proteome both in overall size (714 vs 813 String+ proteins) and in the proportion of each on the Vesiclepedia list (87% vs 85%)

respectively. GO analysis of those proteins that were on neither the EVE list or the Vesiclepedia list showed a strong AD 3-4-specific signal in signal transduction (*GO:0007165*). “Non-specific” recruitment pathway GO terms that were enriched in the non-Vesiclepedia/EVE AD 3-4 and multi AD sets were also enriched to some degree in the demented controls (CCSF) but not in the non-demented control. These included Calcium signaling pathway (*hsa04020*), synaptic transmission (*GO:0007268*), axon (GO:0030424) and dendrite (GO:0030425). This comparison was necessarily restricted to general GO functions by small dataset sizes, but largely confirmed the analysis using the EVE list set.

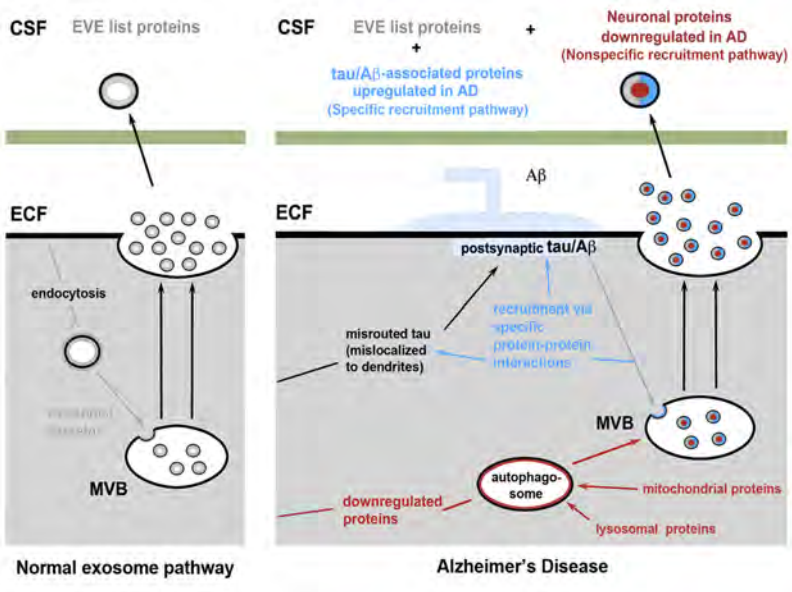

Figure 7: Distinct pathways recruit non-exosomal proteins to exosomes in AD Schematic of peridendritic microvesicle generation (32,97) mechanism illustrates hypothesis for the diversion of up and downregulated proteins to exosomes. Left: The normal exosome pathway causes EVE proteins (shown in gray) to be released with exosome according to the “classical” exosome pathway via the maturation of endosome-derived multivesicular bodies (98). The onset of tauopathy causes a) abnormal local interactions of postsynaptic tau with signaling pathways associated with nonsynaptic functions such as cell cycle control (blue) that may activate or increase cellular utilization of such pathways, resulting in their upregulation and could also cause their diversion to exosomes. This could account for CCRE-associated changes in early AD and may indirectly cause the downregulation of genes that express neuron-specific proteins (red). Such downregulated proteins would not necessarily need to associate directly with tau to be recruited, consistent with their weaker association with tau/Aβ overall as shown in A. Exosomes in early AD might then be expected to be enriched in both upregulated proteins directly recruited to exosomes by interactions with tau or tau associated proteins and downregulated proteins recruited indirectly by CCRE induced de-differentiation.

Discussion Analysis of 3818 exosome-fraction proteins from post mortem CSF samples of 7 patients in early (Braak 3-4) stage AD shows evidence for 2 discrete pathways via which non-exosomal proteins may be recruited to exosomes. Proteins that are upregulated in early to moderate AD are more directly associated with tau and Aβ than other proteins, show a high degree of physical interaction with one another and show a greater degree of participation in pathways that have been shown to mediate the active disruption of signal transduction (19), including events leading to cell cycle re-entry (CCRE) and apoptosis-associated changes in gene expression (18, 27) in cellular AD models. The other enriched group of proteins in the AD 3-4 proteome include proteins that have known neuron-specific functions and that are known to be associated with AD (*hsa05010*). These are largely downregulated in AD and have significantly fewer physical interactions within the group than “upregulated in AD” proteins. The strongest correlation between downregulation in AD and recruitment to exosomes was shown by proteins that are either involved in protein turnover (lysosome) or are components of mitochondria. We interpret this as a diversion of conventional macroautophagy to exosomes, a phenomenon (exophagy) that has been suggested to account for the presence of mitochondrial markers (e.g. mitochondrial ATP synthases, NADH dehydrogenases) in exosomes (23, 31, 54). The enrichment of these terms in neurodegenerative diseases such as AD may therefore simply reflect the increased activity of protein turnover pathways as well as their disruption (55). The evidence for 2 discrete recruitment mechanisms to account for the non-EVE proteome in AD 3-4 CSF exosomes is schematized in Figure 7 and discussed further below.

The AD 3-4 proteome supports a specific hypothesis of early AD neuropathogenesis The characterization of the CSF exosomal proteome in early (Braak stage 3-4) AD presented in this study is consistent with the recently proposed “polarity loss” hypothesis of early AD neuropathogenesis (56-57) that links Aβ-driven synaptotoxicity via postsynaptic tau to CCRE, abortive apoptotic changes, damage to neuronal polarity and ectopic axonogenesis. Toxic interaction between Aβ and tau are prerequisite to AD pathogenesis (47-49) and is thus an early, postsynaptically localized event, where abnormally generated Aβ interacts with a small amount of postsynaptically localized tau (13-15, 17). This interaction has recently been shown to result in the disruption of specific signaling pathways (18-19, 58) and the control of the cell cycle (59), resulting in CCRE (19) and neuronal apoptotic changes similar to those seen in AD. The failure

of cell polarity is a common consequence of carcinogenesis in non-neuronal cell types (60), if (for no other reason) because de-differentiation must precede CCRE in any cell type. We propose that neuronal CCRE in AD induces similar de-differentiating changes in affected neurons that damage neuronal polarity. CCRE in neurons should involve the downregulation of genes that encode neuron-specific functions and structures (covered by the axon, synapse synaptic transmission GO terms). Such changes should lead to the loss of axon-specific targeting of axonal and presynaptic proteins, as has been observed in AD models (14-15, 17, 61) and as a consequence of aging (62). Interestingly, overexpression of tau, which causes the failure of axon-specific sorting at relatively low transgene doses (63-64), causes a highly significant recruitment of downregulated, AD-associated proteins to exosomes in tau-overexpressing neuroblastoma cells (23). Damage to axon-specific localization of tau would then lead the increased localization of tau to the dendrites, producing an amplification of perisynaptic Aβ toxicity (16), and exacerbating the degenerative process. CCRE-induced polarity loss and reactivation of early neurogenesis could also account for the generation of neuropil threads typically seen in AD (7-9) and nonAD tauopathies (65). This hypothesized sequence of events integrates the major cellular features of AD and non AD tauopathies, is consistent with the operation of both of the exosomal recruitment mechanisms described in this study in early AD and is summarized in Figure 8.

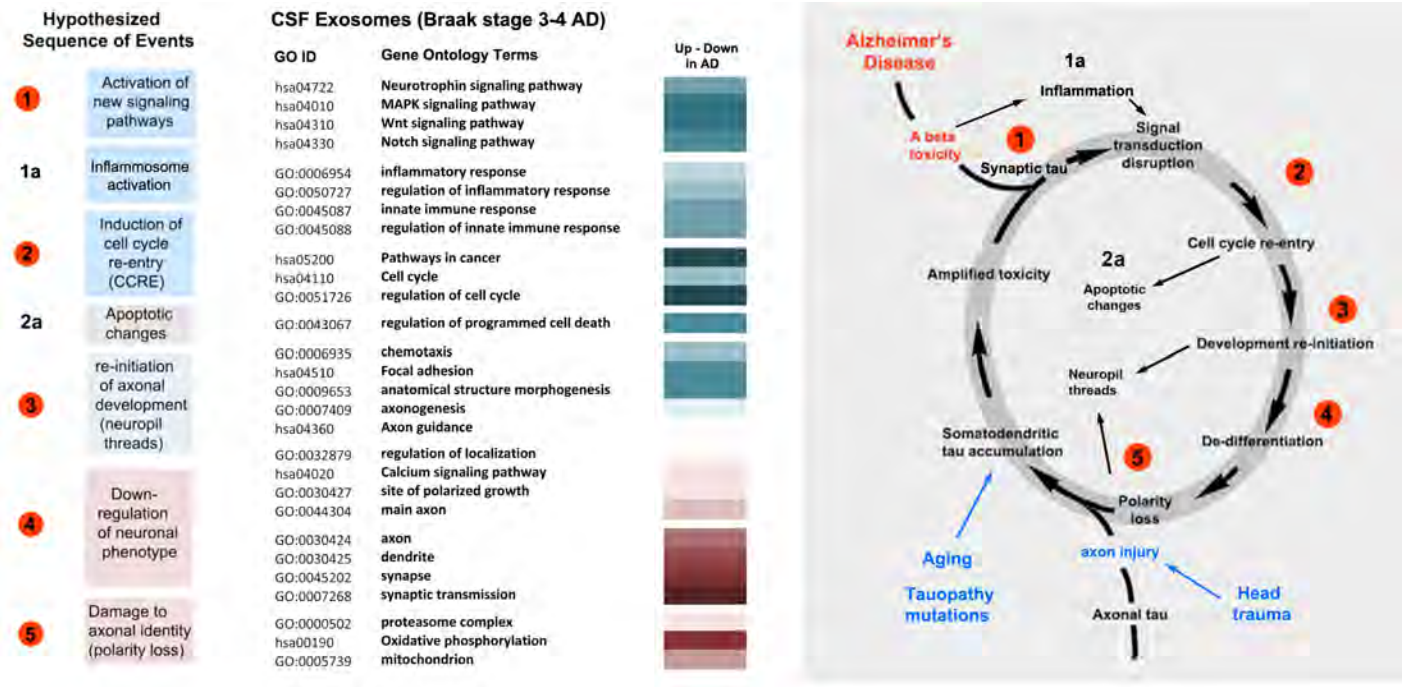

Figure 8 A hypothesis of AD neuropathogenesis that is consistent with the AD 3-4 CSF exosomal proteome accounts for characteristic neuronal features of AD and non-AD tauopathy: The sequence of initial cellular events that we have proposed to account for AD cytopathogenesis are shown at left (red circles) These account for the enrichment of upregulated (blue) and downregulated (pink) in the nonEVE subset of the AD 3-4 proteome. The corresponding GO terms are shown in the heat map, with the degree of enrichment returned by String 9.1 for each term is shown (center). The color reflects the difference in enrichment between up and downregulated (up p value exponent - down p value exponent) proteins for each term, so that The indicated sequence of events is schematized at right and can be summarized as follows: **(1)** Perisynaptic Aβ deposition interacts with postsynaptic tau in plastic neurons to change tau mediated signal transduction, via multiple pathways involving **(1a)** Aβ–induced activation of inflammation (7) and the abnormal activation of kinases such as fyn (16) and GSK3β (28). **(2)** This in turn results in CCRE, causing **(2a)** abortive apoptotic changes (98) and **(3)** the downregulation of the differentiated neuron state. It may also result in synaptic (20) or exosomal (3,23) secretion of tau and associated proteins from dendrites (99-100). Downregulated neuron-specific proteins are shed via the exosome pathway, damaging the axodendritic polarization of the neuron and its ability to efficiently sequester tau to the axon **(4)**, resulting in amplified dendritic toxicity as more tau localizes to the dendrites and is exposed to perisynaptic A beta and enhanced toxicity **(5)**. Exosomal shedding of toxic tau-associated proteins may also occur at this stage. The combination of aberrant axonogenesis and polarity loss results in neuropil thread formation, a characteristic and poorly explained event in tauopathy. Other AD/tauopathy risk factors such as aging-related changes (62), predisposing genetic backgrounds (101), reduced MT binding consequent to tauopathy mutations (102-103) and proximal axonal injury due to head trauma may exacerbate somatodendritic tau accumulation, which amplifies perisynaptic Aβ/tau toxicity (blue arrows) and neurodegeneration.

Evidence for non-specific (clearance driven) and specific (utilization driven) pathways for the recruitment of non-EVE proteins to exosomes The concept of the exosome secretion pathway as a cellular clearance mechanism, or “garbage can pathway”, is well established in the literature, and is prominent in cells that change their function rapidly and frequently, such as cell types involved in the

specific immune response (66-68). The exosome pathway was initially identified and characterized as a mechanism for obsolescent protein turnover (42), but has since had many other functions attributed to it, including homeostatic maintenance (31-33, 69), intercellular communication (22), and the clearance of toxic proteins (70). In the "classical" pathway, the formation of intraluminal vesicles from multivesicular bodies (MVBs) is followed either by secretion as exosomes or fusion with lysosomes, resulting (respectively) in release or degradation (71, 33). It has been suggested that the balance between exosomal or lysosomal fates depends on the dynamics of cell homeostatic requirements (31-33). The release of exosomes can be modulated under conditions that are typically associated with cytotoxicity such as high [Ca2+] (17, 22, 72, 74) or oxidative stress (73). The exosome pathway may also mediate the clearance of Aβ by driving amyloid fibrils to microglia (70), possiblly via autophagic mechanisms (5, 33). Recruitment of the macroautophagy pathway to exosomes ("exophagy") (31) appears to be activated by diverse stimuli, including tau overexpression (23), presenilin insufficiency and the suppression of normal lysosomal function (28). Moreover, it appears to be a feature of multiple neurodegenerative diseases, as mitochondrial markers are prominently associated with Parkinsons Disease (*hsa05012*) and Huntington's Disease (*hsa05016*) as well as AD. The results of this study are consistent with that pattern; we found mitochondrial markers enriched in both the CCSF and AD 3-4 exosomal proteomes. The growing recent acceptance of protein degradation and removal as a normal function of the exosome pathway is epitomized by the recent expansion of the curated "extracellular vesicular exosome" GO term (*GO:0070062*) from 1535 to 2396 members, largely via the addition of lysosomal and (particularly) mitochondrial proteins (51). Since the recruitment of protein turnover pathways to exosomes involves existing cellular pathways that have known mechanisms (e.g. polyubiquitination, KFERQ tags) for identifying and sequestering target proteins, it does not require specific protein-protein interactions and should preferentially target downregulated proteins (23).

Synapse-associated tau/Aβ **interactions may activate specific pathways leading to exosomal secretion** An analysis of our results in the context of a recent characterization of the CSF proteome (50) suggest that the exosome term and signaling proteins in exosomes are upregulated in normal CSF relative to the KB proteome (see Supplemental Figure 1). Moreover, the uptake of exosomes requires energy input (75), suggesting that increased utilization of this pathway in early AD may result in the upregulation of pathway members. The toxic interaction of APP (or Aβ) that underlies much of the later cytopathogenesis of AD is correlated with both upregulated and downregulated functions. APP itself appears to be upregulated in AD, and was present in the CSF exosome fractions of multiple CCSF and AD 3-4 CSF samples, despite the well-established reduction of its toxic cleavage fragment (Aβ) in AD CSF samples. Overall, neuronal tau expression levels are themselves not greatly affected by the onset of AD, but abnormalities in tau distribution that result in increased interaction with synaptic and other signaling mechanisms should result in the upregulation of some of the affected pathways. Increased synaptic plasticity has been reported at the onset of P301L tau induced neurodegeneration in the rTg4510 mouse (13) whereas glutamatergic synaptic activity can modulate the releasing of exosomes from the somatodendritic compartment, suggesting a role for exosome pathway secretion in normal synaptic physiology (22). Finally, increasing evidence suggests that exosome-mediated interneuronal transfer is an important cellular mechanism (36, 40), capable of transmitting tumor promoting factors (34-35) and toxic oligomers that could be involved in neurodegenerative disease propagation (1-4, reviewed by 38, 41). While it is not clear whether specific protein-protein interactions themselves trigger the diversion of non EVE proteins to the exosome pathway in AD, as has been reported for other conditions (25-26, 37), specific alterations to tau have been shown to result in its diversion to exosomes (76), and the exosome pathway does appear to be involved in interneuronal aspects of AD pathogenesis (39). Moreover, it is likely that AD-associated changes that result in oligomerization and/or templated misfolding that lead to interneuronal transfer via exosomes are highly protein and conformation specific. This is especially true for prion disease propagation, where different toxic conformations of the prion protein now appear to mediate the pathogenesis of discrete prion diseases (77) and now appears to be the case with tau as well (78).

Interestingly, GO terms that identify mature neuronal structures and functions are strongly downregulated in AD, as are terms for cellular localization and polarized growth. This is highly suggestive of the shedding of neuronal proteins that would be expected with either CCRE (in AD) or axonal damage resulting in regeneration (in traumatic brain injury syndrome). Both are consistent with the hypothesis that cell polarity loss mediated by tau misprocessing (79) is a central element in the mechanisms mediating AD

cytopathology. The ability of proximal axotomy to cause polarity loss in this way is well documented (80-84), as is the ability of torsional head injury to generate secondary axonal injury foci near the soma in the CNS (85). Destruction of the mechanisms that maintain axon-specific localization (86-88) could therefore account for some of the increased AD risk posed by traumatic injury (89). Depending on the severity of the injury and the presence of other factors such as perisynaptic Aβ (10-12), this is likely to amplify both dendritic degeneration and exosomal release of tau from dendrites in AD and thus possibly cause retrograde spreading of tau lesions as well as NFT formation in the damaged neuron (90). The [Ca++] fluxes due to axonal injury (91) may also produce anterograde transsynaptic lesion spreading as seen in AD (92) and in non-AD tauopathies (93). They may also cause tau to be released to the ventricular and meningeal surfaces of the brain, which may provide a non-synaptic route for tau lesion spreading (90,94).

Summary Exosomes in early AD contain all of the protein elements required to account for the key common features that distinguish AD from most other dementias, including CCRE and CCRE-associated apoptotic changes, neuropil thread growth, somatodendritic tau accumulation, synergy with traumatic injury and (in AD) Aβ-associated synaptopathy. The functional profile of the early AD CSF exosomal proteome reflects all of these disparate features while accounting for gene expression changes associated with AD in the context of a major role for Aβ-driven tau misprocessing in AD cytopathogenesis. We propose that the enrichment pattern that we observed in the AD 3-4 exosomal proteome represents the existence of a causal rather than merely correlative relationship between gene expression changes and CSF exosomal contents in early AD. If so, it may be possible to develop prognostic/diagnostic assays that link body fluid exosome profiles to AD cytopathogenesis in a much more comprehensive and specific way than is true of current assays. It seems likely that one or a group of these proteins will become prominent in the future development of effective diagnostic and/or therapeutic approaches to AD.

Methods All AD 3-4 CSF samples were taken postmortem (post mortem interval < 24h) from patients between the ages of 69-85, diagnosed with clinical AD and neurologically staged at Braak AD stages 3-4 (95). Exosome fractions of these samples yielded 3818 discrete proteins as identified by standard mass spectrometric methods at a 90% identity threshold (3). The main control set used in this study consisted of 1992 discrete proteins identified in exosome fraction samples from 7 demented (VaD/MID) non AD controls with low Braak scores for hippocampal NFTs and no sign of other non AD tauopathy. ELISA data from all of these samples used in this study have been previously published (3); exosome isolation procedures (96) and mass spectrometry analyses are standard and were performed as described (3, 23, respectively). A CSF exosomal proteome consisting of 738 proteins identified by similar methodology (50) was used in some instances.

Expression status in AD The KnowledgeBase (KB) proteome (consisting of 22,258 proteins from the NCBI that were also recognized by String 9.1 (51) was the background superset used for most of the analyses presented (Figure 1). The subsets of the KB proteome that were identified as being either upregulated or downregulated in AD in either late onset (44-45) or familial (43) AD were used for many of the analyses presented here; proteins were listed as given in those studies, both of which reflected changes seen in early AD in array-based analyses of hippocampal and cingulate samples. These studies were congruent with respect to the status of almost all proteins that occurred in both sets; in those cases where there was disagreement, the protein was listed as “unaffected in AD”. This resulted in the identification of 4898 proteins that were “affected by AD” (2492 up, 2406 down see Figure 1a). a more complete description of these subsets can be found in Saman (23).

Exosome list (EVE) proteins We defined these as the 2396 proteins (gene products) that were recognized by String from the GO term “extracellular vesicular exosome” *(GO:0043230)*. These were essentially a subset of the 2973 proteins identified by citation in Exocarta (23), with most of the proteins in the ExoCarta list that were excluded from the EVE list being in proteins with low (1-2 cites) ExoCarta cite rates. The GO term set was used in all of the analyses presented here.

Physical interactions with tau and Aβ In order to assess the relationship between the CSF exosomal proteome and cytopathological events associated with the onset of AD, we used String 9.0 to subdivide the

KnowledgeBase proteome (46) of 22,258 into subsets based on their direct and indirect physical interactions with both tau and Aβ using a reiterative query method similar to that described (23). This method generated a ranked list of proteins based on increasingly indirect physical associations with APP and tau. Setting String interaction parameters to "Experiments" only and using the maximum setting for interactors (500), we added the results of search for MAPT and APP obtained 404 physically interacting proteins in the String database, or 2% of the KB proteome. These were then added to tau and the search was re-iterated as needed using a confidence level of 0.7 (high). We subdivided the KnowledgeBase set based of physical association with tau, with the strongest direct (first iteration) and indirect (second and later iterations) physical interaction scores with a list consisting of MAPT and APP). The top 10% = 2200 proteins most directly associated with MAPT and APP, the next 20% = the next 4400 proteins, the remaining 15,659 of the KB set = the bottom 70% of proteins. In some analyses (see Figure 6), the top 2% of all String/KB+ proteins was used as well. In all cases, category membership was assigned according to the iteration number (with each screening identifying up to 500 proteins most strongly physically associated with the results of the previous screen) followed by the physical association score (within the last iteration list required to reach the target percentage desired (i.e. 2%, 10%, 30%).

Statistical analysis of connectivity in Figure 2b, the 1" boxes (shaded) were placed so as to maximize icon inclusion (black dots) for each 1.9" diagram shown. The percentage of pathway members covered by the box (shown above each diagram) is a measure of physical and functional interaction (51). The ratio between the box and total diagram areas is normalized to 28%, which is the percentage of icon coverage expected with a completely random icon distribution.

The very large (15,659) group of least MAPT/APP-associated proteins (i.e. the bottom 70% of all KB proteins) included many poorly characterized proteins, including those identified as chromosomal open reading frames, those identified by KIA and ENG numbers, etc, and was defined by exclusion criteria only, unlike the other categories described. In order to prevent these features from introducing distortions into analyses of specific protein-protein interactions and (in some cases) to facilitate String-based analyses (String cannot process batches of gene name lists longer than 2000 queries), these proteins were excluded when circumstances permitted, and a comparison between the top 10% and next 20% of proteins was substituted for them, allowing a categorical analysis and characterization of the AD 3-4 and CCSF proteomes based on the relative enrichment/depletion of AD associated pathways and cellular functions among proteins known to have some degree of direct or indirect physical interaction with APP and/or tau using String 9.1.. Parallel calculations based on the entire KB dataset were also (when possible) and are presented in the Supplementary File 2.

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	6019 csf 4	Score 77	6019 B4	Score 77		10457 CSF4	Score 66
	Up	Down	Up	Down		Up	Down
Total	110	26	32	34		655	143
nonEVE	80	24	27	28		521	99
Set 1 (nonEVE)	24	4	7	2		147	21
Set 1+2 (nonEVE)	44	15	15	16		323	56
EVE	30	2	5	6		134	44
Set 1 (EVE)	4	2	2	1		33	9
Set 1+2 (EVE)	19	2	3	5		89	32
% nonEVE	73%	92%	84%	82%		80%	69%
% Set 1 (nonEVE)	22%	15%	22%	6%		22%	15%
%Set 1+2 (nonEVE	55%	63%	56%	57%		62%	57%
% EVE	27%	8%	16%	18%		20%	31%
% Set 1 (EVE)	13%	100%	40%	17%		25%	20%
%Set 1+2 (EVE)	63%	100%	60%	83%		66%	73%

		CSF				HC nonEVE	
	Up	% nonEVE	% Set 1	% Set 1+2		% nonEVE	% Set 1
6019 c	Score 77	73%	22%	55%		84%	22%
10457	Score 66	80%	22%	62%		84%	26%
6055 c	Score 63	79%	25%	64%		83%	18%
10226	Score 56	79%	19%	50%		74%	26%
6033 c	Score 46	84%	19%	52%		83%	15%
	Average	**79%**	**22%**	**57%**		**82%**	**21%**
	Down						
6019 c	Score 77	92%	15%	63%		82%	6%
10457	Score 66	69%	15%	57%		80%	8%
6055 c	Score 63	83%	18%	62%		79%	16%
10226	Score 56	74%	15%	55%		91%	22%
6033 c	Score 46	91%	27%	50%		83%	10%
	Average	**82%**	**18%**	**57%**		**83%**	**12%**
	Up/Down						
6019 c	Score 77	0.79	**1.42**	0.88		1.02	**3.72**
10457	Score 66	1.15	**1.53**	1.10		1.05	**3.23**
6055 c	Score 63	0.96	**1.36**	1.04	**2.86E-02** 77-66-63	1.06	1.18
10226	Score 56	1.07	1.30	0.91		0.81	1.20
6033 c	Score 46	0.93	0.69	1.04	NS	1.00	1.54
	Average	**0.98**	**1.26**	**0.99**		**0.99**	**2.17**

% EVE/nonEVE doesn't vary with either CSF/HC or with E2- score

Proportion Up/down doesn't vary with % EVE or %Set 1 vs E2- in CSF

It DOES in

Proportion of "down in AD" Set 1 proteins in HC (EVE and nonEVE) increases with decreasing E2
% Set 1 in CSF EVE, but not HC increases with DECREASING E2- score

10457 B4	Score 66		6055 csf 3	Score 63		6055 B3	Score 63	
Up	Down		Up	Down		Up	Down	
31	25		131	92		473	461	
26	20		104	76		393	362	
8	2		33	17		87	72	
16	10		67	47		193	171	
5	5		27	16		80	99	
2	1		8	2		28	24	
3	4		16	10		62	65	
84%	80%		79%	83%		83%	79%	
26%	8%		25%	18%		18%	16%	
62%	50%		64%	62%		49%	47%	
16%	20%		21%	17%		17%	21%	
40%	20%		30%	13%		35%	24%	
60%	80%		59%	63%		78%	66%	

		CSF EVE				HC EVE	
% Set 1+2		% EVE	% Set 1	% Set 1+2	Set 1 vs Set 2	% EVE	% Set 1
56%		27%	13%	63%		16%	40%
62%		20%	25%	66%	**2.91E-03** 77-66	16%	40%
49%		21%	30%	59%	**4.64E-03** 77-66-63	17%	35%
65%		21%	33%	67%		26%	50%
41%		16%	40%	60%		17%	43%
54%		**21%**	**28%**	**63%**		**18%**	**42%**
57%		8%	100%	100%		18%	17%
50%		31%	20%	73%		20%	20%
47%		17%	13%	63%		21%	24%
57%		26%	57%	100%		9%	50%
40%		9%	100%	100%		17%	57%
50%		**18%**	**58%**	**87%**		**17%**	**34%**
0.97	0.0538	3.55	0.13	0.63		0.89	**2.40**
1.23		0.66	1.20	0.91		0.81	**2.00**
1.04		1.19	2.37	0.95		0.79	1.44
1.13		0.81	0.58	0.67		3.00	1.00
1.03		1.72	0.40	0.60		1.02	0.75
1.08		**1.59**	**0.94**	**0.75**		**1.30**	**1.52**

ıcrease with E2- relative to Set 2, esp if use CSF +HC

FET all E2- vs E2+							FET all E2- vs E2+			
Union N term and full length tau			Up	Down	FET vs All			Set 1	Set 2	FET vs All
8.28E-02	**Inflammation**	**E2-**	18	30	NS		NS	38	37	**1.39E-02**
		E2+	7	3	NS			11	9	NS
1.07E-02	**Synapse**	**E2-**	9	74		**7.14E-05**	NS	42	42	**1.01E-02**
		E2+	3	9		**2.85E-02**		8	8	NS
2.50E-02	**Secretion**	**E2-**	7	51		**1.88E-03**	NS	33	39	NS
		E2+	6	5	NS			5	10	NS
		E2-	144	344		**2.33E-09**		196	328	NS
		E2+	78	57				73	98	
Full length tau isoforms										
1.14E-02	**Signaling**	**E2-**	13	23	NS		NS	30	10	**2.59E-07**
		E2+	5	0		*6.49E-02*		7	4	*9.33E-02*
NS	**Inflammation**	**E2-**	16	29	NS		NS	22	34	NS
		E2+	2	2	NS			7	4	*9.33E-02*
NS	**plasticity**	**E2-**	3	31		**1.41E-02**	NS	26	14	**1.41E-04**
		E2+	1	1	NS			3	1	NS
NS	**AD**	**E2-**	7	39		*7.67E-02*	NS	25	24	**2.89E-02**
		E2+	1	2	NS			1	3	NS
NS	**Synapse**	**E2-**	8	72		**2.53E-04**	NS	40	40	**1.64E-02**
		E2+	1	3	NS			3	4	NS
NS	**Secretion**	**E2-**	6	47		**7.28E-03**	NS	41	35	**1.96E-03**
		E2+	2	2	NS			2	6	NS
NS	**Turnover**	**E2-**	5	60		**2.44E-04**	NS	18	47	*7.30E-02*
		E2+	2	5	NS			2	8	NS
6.43E-07	**Total full length**	**E2-**	113	308			NS	166	282	
		E2+	43	33				38	66	
N terminal tau fragments (1-255)										
NS	**Signaling**	**E2-**	2	3	NS		NS	7	2	**1.75E-02**
		E2+	2	1	NS			7	2	**5.70E-03**
NS	**Inflammation**	**E2-**	7	2		*9.09E-02*	NS	10	7	NS
		E2+	4	2	NS			3	6	NS
NS	**plasticity**	**E2-**	1	2	NS		NS	6	1	**1.37E-02**
		E2+	0	2	NS			2	1	NS
NS	**AD**	**E2-**	1	6	NS		NS	2	5	NS
		E2+	1	2	NS			2	2	NS
NS	**Synapse**	**E2-**	2	9		**5.31E-02**	NS	6	6	NS
		E2+	1	3	NS			2	3	NS
NS	**Secretion**	**E2-**	2	8	NS		NS	4	8	NS
		E2+	4	1	NS			2	5	NS

NS	**Turnover**	**E2-**	5	10	NS	NS	7	8	NS	
		E2+	3	3	NS		2	8	NS	
NS	**Total N terminal ta**	**E2-**	55	57	NS	NS	52	82		
		E2+	44	29			31	62		

Exosomal fraction proteins in the
are significantly more tau/Abeta-
Both full length and N terminal cc
presence in Set 1 (the top 10% of

There is no difference in the prop
between E2- and E2+ exosomal fr

Signaling and inflammation are th
Neuronal functions are downregu
Turnover mechanisms are least ta

This pattern resembles that seen
and is not affected by E2+ vs -

N terminal fragments still have to
However, they also appear to exh

Full length E2- tau is associated w
E2+ tau is not, but the small samp
There is no apparent difference ir

FET N terminal vs full length vs other pathways

Up vs down in AD	Tau association
NS	NS
NS	NS
2.82E-02	NS
NS	NS
NS	NS
NS	NS
NS	NS
NS	NS
NS	NS
NS	NS
NS	NS
NS	NS

Inflammation associated proteins
down in AD with E2- full length, b
This is especially so when compar
8.02E-04

It is tempting to suppose that E2-
tau toxicity than other isoforms, s
nonspecific recruitment pathway

NS	NS	
NS	NS	The widespread upregulation of C

EVE GO term from E2- tau expressing SY5Y cells
associated than are those from E2+ expressing cells
onstructs show a 2.5 fold greater
proteins by tau/Abeta links)

ortion of EVE and recruited (non-EVE) proteins
actions

EVE (exosome GO term)
% EVE

up in AD
down in AD

Set 1 (tau associated)
Set 2

ne most upregulated pathways
ulated but still tau associated
au associated

FET E2- isoforms more tau associated

in early AD CSF exosomal proteome

o small a sample size for conclusions
ibit the above pattern

with downreg proteins in exosomes
ole size precludes definite conclusions
n the degree of tau association

s are
ut not N terminal
ed to signaling pathways generally

tau may be more closely associated with
since toxicity is a hallmark of the

CSF exosome proteins In AD is not reflected by E2- and E2+ tau overexpression

Full length		**N terminal**		All	
E2-	**E2+**	**E2-**	**E2+**	**E2-**	**E2+**
594	148	194	138	788	286
118	25	23	27	141	52
20%	17%	12%	20%	**18%**	**18%**
17	5	8	11	25	16
70	6	5	6	75	12
87	11	13	17	100	28
20%	45%	62%	65%	25%	57%
47	3	8	4	55	7
51	14	9	15	60	29
98	17	17	19	115	36
48%	18%	47%	21%	48%	19%
3.18E-02		*1.58E-01*		**3.25E-03**	